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(54) Title: ANTISENSE OLIGOMERS FOR INHIBITING HUMAN PAPILLOMAVIRUSES

(57) Abstract

Antisense oligomers complementary to and capable of hybridizing to a target region of an mRNA or pre-mRNA of a human papillomavirus are provided. Suitable target regions include sequences selected from a translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a polyadenylation signal, a 3'-untranslated region or a 5'-untranslated region of an HPV gene.

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# DESCRIPTION

## Antisense Oligomers For Inhibiting Human Papillomaviruses

### Related Applications

This application is a continuation-in-part application of commonly-assigned United States Patent Application Serial No. 08/350,431, filed December 5, 1994; which is a continuation-in-part of commonly owned PCT application PCT/US94/13387 filed November 16, 1994; which is a continuation-in-part of commonly-assigned United States Patent Application Serial No. 08/238,177, filed May 4, 1994; which is a continuation-in-part of United States Patent Application Serial No. 08/233,778, filed April 26, 1994; which is a continuation-in-part of United States Patent Application Nos. 08/154,013 and 08/154,014, both filed November 16, 1993. The disclosures of all these applications are incorporated by reference.

### Background of the Invention

Papillomaviruses are a group of small DNA viruses that induce warts (or papillomas) in a number of higher vertebrates, including humans. Although the viral nature of human warts has been known for many years, it has only recently been recognized that specific human papillomavirus ("HPV's") are closely linked with certain human cancers most notably human cervical carcinoma. This finding has focused interest on the specific subgroup of HPV's associated with genital infections.

The papillomaviruses are reported to be highly species specific and to induce squamous epithelial

tumors and fibroepithelial tumors in their natural hosts.

Bovine papillomavirus-1 (BPV-1) has been more fully characterized and has served as a prototype for studies on the transformation and molecular biology of human papillomaviruses. Significant differences exist between BPV-1 and the HPV's.

Certain proteins have been proposed as targets for antisense therapy of HPV-caused conditions. Certain of these targets have been proposed based on studies performed using BPV-1 and equivalent functions and properties have not necessarily been confirmed in all HPV's. In addition, other parameters to be considered in the selection of targets for antisense effect, such as mRNA sequence homology of target proteins between BPV and a HPV or the mRNA's secondary structure (which strongly affect the accessibility of the target sequences) are low or are not known. This makes selection of HPV target sequences based on BPV target sequences almost impossible.

HPV's associated with a risk for malignant progression include HPV-16, HPV-18, HPV-31 and HPV-33, among others. Genital HPV's not associated with a risk for malignant progression include HPV-6b and HPV-11.

In human papillomaviruses the E7 protein has been reported to have transcriptional modulating properties and to complex p105-RB, a product of the retinoblastoma tumor suppressor gene. In HPV-16 and HPV-18, E7 is reported to encode transforming proteins, proteins which are multifunctional and possess both transcriptional modulatory and transformation properties similar to that adenovirus EIA. It has been reported that the E7 proteins of all the genital associated HPV's can complex p105-RB <u>in vitro</u> regardless of their associated risk for malignant progression. It has been proposed that the ability of the E7 proteins to complex p105-RB must

relate to a property common both to the high risk viruses (such as HPV-16 and HPV-18) and the low risk virus (such as HPV-6b and HPV-11), such as the induction of cellular proliferation, a feature of all benign warts. In addition, since not all E7 proteins of the genital papillomaviruses can transform in tissue culture, it may be that the ability to complex p105-RB is not sufficient for transformation. Moreover, the mechanism by which E7 modulates transcription has not been identified. (Fields, Virology, Vol. 2, p. 1639 (1990)).

The E6 gene and its protein is also reported as associated with transformation in high risk human papilloma viruses, through interactions with p53. (Band, et al., EMBO Journal, Vol. 12, No. 5, pp. 1847-1852 (1993)).

Although certain HPV's such as HPV-6 and HPV-11 have been categorized as "low risk", in that patients infected with these HPV's appear to be at significantly lower risk for malignant progression, it should be noted that such infections are not without risk for malignant progression and occasional tumors do contain these viral genomes that are transcriptionally active. Therefore, all genital HPV's should be considered capable of causing serious conditions. Accordingly, detection and control of HPV infections, especially those caused by genital HPV's is important.

## Summary of the Invention

The present invention is directed to antisense oligomers which are complementary to and which are capable of hybridizing with a target sequence of a mRNA or pre-mRNA of an human papillomavirus. In particular, provided are antisense oligomers which are complementary to and which hybridize with a portion of a mRNA or pre-mRNA encoding E1, E2, E6 or E7 gene of an HPV. These

antisense oligomers interfere with and/or prevent expression of their target mRNA and thus, may be used for treatment and/or diagnosis of HPV infections as well as for research purposes.

Accordingly, provided are oligomers complementary to a target sequence which is a portion of a HPV mRNA or pre-mRNA which encodes E1, E2, E6 or E7. Preferably, the target sequence is in the region of the initiation codon, more preferably from about -25 to about +35 relative to the initiation codon where the initiation codon is +1 to +3.

Definitions

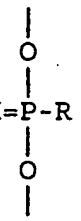
As used herein, the following terms have the following meanings unless expressly stated to the contrary.

The term "purine" or "purine base" includes not only the naturally occurring adenine and guanine bases, but also modifications of those bases such as bases substituted at the 8-position, or guanine analogs modified at the 6-position or the analog of adenine, 2-amino purine, as well as analogs of purines having carbon replacing nitrogen at the 9-position such as the 9-deaza purine derivatives and other purine analogs.

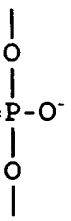
The term "pyrimidine" or "pyrimidine base", includes not only the naturally occurring cytosine, uracil and thymine but also modifications to these bases such as 5-propynyluracil, 5-heteroaryluracils and analogs of pyrimidines such as reported heteroaromatic moieties.

The term "nucleoside" includes a nucleosidyl unit and is used interchangeably therewith, and refers to a subunit of a nucleic acid which comprises a 5-carbon sugar and a nitrogen-containing base. The term includes not only those nucleosidyl units having A, G, C, T and U as their bases, but also analogs and modified forms of the naturally-occurring bases, including the pyrimidine-

analogs such as pseudoisocytosine and pseudouracil and other modified bases (such as 8-substituted purines). In RNA, the 5-carbon sugar is ribose; in DNA, it is a 2'-deoxyribose. The term nucleoside also includes other analogs of such subunits, including those which have modified sugars such as 2'-O-alkyl ribose.

The term "phosphonate" refers to the group 

wherein X is oxygen or sulfur, R is hydrogen or an alkyl or aryl group, and thus includes various example of phosphonate and phosphonothioate internucleosidyl linkages. Suitable alkyl or aryl groups include those which do not sterically hinder the phosphonate linkage or interact with each other. The phosphonate group may exist in either an "R" or an "S" configuration. Phosphonate groups may be used as internucleosidyl linkages (or links) to connect nucleosidyl unit or a nucleosidyl unit and a non-nucleosidy monomeric unit. The term "lower alkylphosphonate" refers to groups where X is oxygen and R is lower alkyl of 1 to 3 carbon atoms. "Methylphosphonate" refers to groups where X is oxygen and R is methyl. The term "phosphonothioate" refers to those groups where X is sulfur. The term "lower alkylphosphonothioate" refers to groups where X is sulfur and R is lower alkyl of 1 to 3 carbon atoms. The term "methylphosphonothioate" refers to a phosphonothioate group wherein R is methyl.

The term "phosphodiester" or "diester" refers to the group  wherein phosphodiester groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

A "non-nucleoside monomeric unit" refers to a monomeric unit wherein the base, the sugar and/or the phosphorus backbone has been replaced by other chemical moieties.

A "nucleoside/non-nucleoside polymer" refers to a polymer comprised of nucleoside and non-nucleoside monomeric units.

The term "oligonucleoside" or "Oligomer" refers to a chain of nucleosides which are linked by internucleoside linkages which is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are usually synthesized from nucleoside monomers, but may also be obtained by enzymatic means. Thus, the term "Oligomer" refers to a chain of oligonucleosides which have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic oligonucleoside alkyl- and aryl-phosphonate analogs, alkyl- and aryl-phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleoside analogs, such as phosphotriesters and other oligonucleoside analogs and modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The term also includes nucleoside/non-nucleoside polymers wherein one or more of the phosphorus group linkages between

monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a sulfamate linkage, a carbamate linkage, an amide linkage, a guanidine linkage, a nitroxide linkage, or a substituted hydrazine linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as morpholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers wherein the base, the sugar, and the phosphate backbone of the non-nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-nucleoside moiety may serve to link other small molecules which may interact with target sequences or alter uptake into target cells.

The term "neutral Oligomer" refers to Oligomers which have nonionic internucleosidyl linkages between nucleoside monomers (i.e., linkages having no positive or negative ionic charge) and include, for example, Oligomers having internucleosidyl linkages such as alkyl- or aryl- phosphonate linkages, alkyl- or aryl-phosphonothioates, neutral phosphate ester linkages such as phosphotriester linkages, especially neutral ethyltriester linkages; and non-phosphorus-containing internucleosidyl linkages, such as sulfamate, morpholino, formacetal, thioformacetal, and carbamate linkages. Optionally, a neutral Oligomer may comprise a conjugate between an oligonucleoside or nucleoside/non-nucleoside polymer and a second molecule which comprises a conjugation partner. Such conjugation partners may comprise intercalators, alkylating agents, binding substances for cell surface receptors, lipophilic agents, nucleic acid modifying groups including photo-

cross-linking agents such as psoralen and groups capable of cleaving a targeted portion of a nucleic acid, and the like. Such conjugation partners may further enhance the uptake of the Oligomer, modify the interaction of the Oligomer with the target sequence, or alter the pharmacokinetic distribution of the Oligomer. The essential requirement is that the oligonucleoside or nucleoside/non-nucleoside polymer that the Oligomer conjugate comprises be substantially neutral.

The term "substantially neutral" in referring to an Oligomer refers to those Oligomers in which at least about 80 percent of the internucleosidyl linkages between the nucleoside monomers are nonionic linkages.

The term "acid resistant" refers to Oligomers which are resistant, in comparison to deoxyribooligonucleotides, to acid-catalyzed depurination by hydrolysis of the N-glycosyl bond.

The term "Triplex Oligomer Pair" refers to first and second Oligomers which are optionally covalently linked at one or more sites and which are complementary to and are capable of hydrogen bonding to a segment of a single stranded target nucleic acid, such as RNA or DNA, and, thus, together with the single stranded target nucleic acid, are capable of forming a triple helix structure therewith.

The term "Third Strand Oligomer" refers to Oligomers which are capable of hybridizing to a segment of a double stranded nucleic acid, such as a DNA duplex, an RNA duplex or a DNA-RNA duplex, and forming a triple helix structure therewith.

The term "complementary," when referring to a Triplex Oligomer Pair (or first and second Oligomers) or to a Third Strand Oligomer, refers to Oligomers having base sequences which are capable of forming or recognizing hydrogen bonds (and base pairing or

hybridizing) with the base sequence of the nucleic acid to form a triple helix structure.

The term "substantially complementary" refers to Oligomers, including Triplex Oligomer Pairs or Third Strand Oligomers which may lack a complement for each nucleoside in the target sequence, have sufficient binding affinity for the target sequence to form a stable duplex or triple helix complex, as the case may be, and thereby specifically recognize the target sequence and selectively inhibit or down-regulate its expression.

The term "triplet" or "triad" refers a hydrogen bonded complex of the bases of three nucleosides between a base (if single stranded) or bases (if double stranded) of a target sequence, a base of a Second Strand and a Third Strand (if a single stranded target sequence) or a base of a Third Strand (if a double-stranded target).

"MP(Rp)" refers to a methylphosphonate internucleosidyl linkage of Rp chirality.

"MPS" refers to a methylphosphonothioate internucleosidyl linkage.

"MPS(Rp)" refers to a methylphosphonothioate internucleosidyl linkage of Rp chirality.

An oligomer having "alternating MP(Rp)/DE internucleosidyl linkages" refers to an Oligomer wherein methylphosphonate linkages of Rp chirality alternate with phosphodiester linkages ("DE").

An oligomer having "alternating MP(Rp)/PS internucleosidyl linkages" refers to an oligomer wherein methylphosphonate linkages of Rp chirality alternate with phosphorothioate linkages ("PS").

An oligomer having "alternating MPS(Rp)/DE internucleosidyl linkages refers to an oligomer wherein methylphosphonothioate linkages of Rp chirality alternate with phosphodiester linkages.

An oligomer having "alternating MPS(Rp)/PS internucleosidyl linkages" refers to an oligomer wherein methylphosphonothioate linkages of Rp chirality alternate with phosphorothioate linkages.

A "MP(Rp)/DE dimer synthon refers to a dinucleoside wherein the two nucleosides are linked by a methylphosphonate internucleosidyl linkage of Rp chirality and one of the nucleosides has a 5'- or 3'- coupling group which when coupled to a 3'-OH or a 5'-OH, of another nucleoside or an oligomer will result in a phosphodiester internucleosidyl linkage.

A "MP(Rp)/PS dimer synthon" refers to a dinucleoside wherein the two nucleosides are linked by a methylphosphonate linkage of Rp chirality and one of the nucleosides has a 5'- or 3'- coupling group which when coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphorothioate internucleosidyl linkage.

A "MPS(Rp)/DE dimer synthon" refers to a dinucleoside wherein the two nucleosides are linked by a methylphosphonothioate linkage of Rp chirality and one of the nucleosides has a 5'- or 3'- coupling group which when coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphodiester internucleosidyl linkage.

A "MP(Rp)/$PS_2$ dimer synthon" refers to a dinucleoside wherein the two nucleosides are linked by a methylphosphonate linkage of Rp chirality and one of the nucleosides has a 5'- or 3'- coupling group which when coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphorothioate internucleosidyl linkage.

A "2'-O-methyl MP(Rp)/2'-O-methyl DE dimer synthon" refers to a dinucleoside wherein two 2'-O-methyl nucleosides are linked by a methylphosphonate linkage of Rp chirality and one of the nucleosides has a 5'- or 3'-

coupling group which when coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphodiester internucleosidyl linkage.

Brief Description of the Drawings

Figures 1A and 1B depict representation the structures of polycistronic E6/E7 mRNA (Figure 1A) and monocistronic E7 mRNA (Figure 1B) found in HPV-6b or HPV-11 condylomas. The mRNA structures shown are according to Smotkin et al. (J. Virol 63, 1441-1447). The E6/E7 and E7 transcripts are depicted as solid lines. The closed circles at the 5' ends represent the putative promoters. The coding regions for E6 and E7 are indicated by open (E6) or shaded (E7) boxes superimposed on the mRNAs. The nucleotide position of the translation initiation codons and termination codons for E6 and E7 are indicated.

Figures 2A and 2B depict phylogenetic analysis of the secondary structure of HPV E7 mRNA around the translation initiation codon. Figure 2A depicts alignment of HPV sequences around the E6/E7 boundary. Initial sequence alignments were made with Clustal 3 (Higgins et al., CABIOS 5:151-153 (1989)). Further alignments were made by hand. Secondary structural elements were generated by RNAfold 2 (Scientific and Educational Software), and PCfold 4 (Zuker et al., Nucleic Acids Res. 9:133-148 (1981)). Numbers above HPV-11 are the sequence numbering for HPV-11. Numbers in brackets are extra bases between secondary structural element and the AUG. "cons # 1" and "cons # 2" are the conserved bases for this alignment (R = G, A, Y = U, C). Lower case letters indicate bases conserved in the majority of sequences studied. Sequence elements that are helical are shown in boldface. Periods indicate the base at that position is not contained. Figure 2B depicts a schematic representation of the pseudoknot

structure present upstream of the initiation codon of E7 and also a detailed representation of the pseudoknot sequence found in HPV-11 and in HPV-6b.

Figures 3A and 3B depict inhibition of cell-free translation of monocistronic E7 in RNA with RNase H mediated cleavers (Figure 3A) or with steric blockers (Figure 3B). HPV-11 E7 monocistronic mRNA (circles) or E6/E7 polycistronic mRNA (squares) were translated in rabbit reticulocyte lysates in the absence or in the presence of different concentrations of [MP] $[DE]_5$ [MP] oligonucleotide 2567-1 [SEQ. ID. NO. 26], (Figure 3A) or 2'OMeRNA oligonucleotide 2644-1 [SEQ. ID. NO. 18] (Figure 3B). Translation reactions were carried out at 37°C. RNAse H at 0.04 units/ul was present in the translations run in the presence of oligonucleotide 2567-1. CAT mRNA at 10 nM, was co-translated as negative control in both cases (triangles). The effect of the oligomers on E7 translation was evaluated after immuno-precipitation of the translation reaction with αE7 anti-serum, size fractionation on SDS PAGE and phospho-image analysis. The effect of the oligomer on CAT translation was evaluated after size fraction on SDS PAGE gel on one aliquot of the reaction mixture followed by phospho-image analysis. Results are expressed as percent of protein translation with respect to the value of translation obtained in the absence of oligonucleotides.

Figures 4A and 4B depict inhibition of E7 and E6 in cell-free synthesis with RNAse H mediated cleavers: phosphodiester oligomer 2498 [SEQ. ID. NO. 14] (Figure 4A) or methylphosphonate end-capped diester oligomer 2567-1 [SEQ. ID. NO. 26] (Figure 4B). HPV-11 E6/E7 polycistronic mRNA was translated in rabbit reticulocyte lysates in the absence or in the presence of different concentrations of phosphodiester oligonucleotide 2498-1 (Figure 4A) or [MP] $[DE]_5$ [MP] oligonucleotide 2567-1,

(Figure 4B). Translation reactions were carried out at 37°C, in the presence of RNAse H at 0.04 units/ul. CAT mRNA, at 10 nM, was co-translated as negative control in both cases. The effect of the oligomers on E7 translation (circles) was evaluated after immuno-precipitation of the translation reaction with αE7 anti-serum, size fractionation on SDS PAGE and phospho-image analysis. The effect of the oligomer on E6 translation (squares) or CAT translation (triangles) was evaluated after size fractionation on SDS PAGE gel of one aliquot of the reaction mixture followed by phospho-image analysis. Results are expressed as percent of protein translation with respect to the value of translation obtained for each protein in the absence of oligonucleotides.

Figure 5 depicts inhibition of transient expression of E7 in COS-7 cells with [Rp-MP/DE] $[PS]_5$ [Rp-MP/DE] oligomer 3256-1 [SEQ. ID. NO. 32]. E7 expression plasmid pcDNA11E7 (5 ug/ml) and different amounts of antisense oligonucleotide were transfected COS-7 cells in the presence of Transfectam™ (Promega). Cells were incubated with transfection mixture for 4 hours, allowed to recover in media plus serum overnight, and labeled with $^{35}S$-cysteine for 5 hours before harvesting. Cells were lysed and E7 protein synthesis was evaluated by immuno-precipitation with αE7 serum followed by SDS-PAGE gel fractionation or protein products and phospho-image analysis. Total protein synthesis was analyzed by SDS-PAGE separation of an aliquot of the cell extract, autoradiography and phospho-image quantitation of all the proteins present in each lane. Results are expressed as percentage of protein translation respect to the value of translation obtained in the absence of oligonucleotide.

Figures 6A and 6B depict reduction of E7 mRNA levels in COS-7 cells treated with [Rp-MP/DE] [PS] [Rp-MP/DE]

oligomer 3256 [SEQ. ID. NO. 32]. E7 expression plasmid pcDNA11E7 (5 ug/ml) alone (lane 1) or together with 5 uM of control oligomer 3215-1 [SEQ. ID. NO. 76] (lane 5) or 0.05 uM (lane 2), 0.5 uM (lane 3) or 5 uM (lane 4) of oligomer 3256-3 [SEQ. ID. NO. 32], was transfected into COS-7 cells using Lipofectamine™ (BRL). Twenty four hours after the transfection, intracellular RNA was extracted and RNase protection assays were carried out using an HPV-11 E7 $^{32}$P-labeled RNA probe (Figure 6A) or a GAPDH $^{32}$P-labeled RNA probe (Figure 6B).

Figure 7 depicts inhibition of expression of E6 and E7 proteins in cells by chimeric methylphosphonate oligonucleotide 3256. Expression plasmids encoding E6 and E7 were transfected into COS-7 cells together with oligonucleotide 3256-3 [SEQ. ID. NO. 32] at 5 uM. Cells were metabolically radiolabelled the next day and immune precipitates were prepared and analyzed by PAGE and autoradiography as described in Figure 5. Oligonucleotide 3256 targets nucleotides 523-542 of HPV-11, corresponding to the 5' end of the E7 ORF and the 3' end of the E6 ORF. Control oligonucleotide 3218 [SEQ. ID. NO. 46] targets E1.

Figure 8 depicts coding potentials of the HPV-11 E-region transcripts. The circular genome is represented in a linear form with the ORFs (open boxes) and their possible functions indicated above. Vertical dashed lines inside each ORF mark the location of the first AUG codon. All viral E-region transcripts are depicted as arrows in the 5'-to-3' direction, with gaps representing introns spliced out of the transcripts and numbers indicating the nucleotide positions of exon boundaries adjacent to splice donors and acceptors. The closed circle at the 5' end of each message represents the proven or putative promoter, and the arrowheads at the 3' ends designate the polyadenylation sites. Coding potentials, as deduced from the cDNA sequences, are

drawn as open boxes superimposed on each mRNA arrow, and the encodes proteins are named at the 3' end of each transcript.

Figures 9A to 9C depict structures of E6/E7 transcripts found in HPV-16 transformed CaSki cells. The mRNA structures shown are according to Smotkin and Wettstein (Proc. Natl. Acad. Sci. USA 83, 4680-4684). Figure 9A depicts E6/E7 mRNA. Figure 9B depicts E6*I/E7 mRNA. Figure 9C depicts E6*II/E7 mRNA. The E6/E7, E6*I/E7 and E6*II/E7 transcripts are depicted as solid lines. The introns resulting from two alternative splicing events within the E6 ORF are depicted as dashed lines, the closed circles at the 5' ends represent the putative promoters. The coding regions for E6, E6*I, E6*II and E7 are indicated by light (E6) or dark (E7) shaded boxes superimposed on the mRNAs. The nucleotide position of the translation initiation codons, termination codons for E6 and E7, and splice donor and acceptor sites within E6 are indicated.

Figure 10 depicts reduction of E7 mRNA levels in HPV transformed CaSki cells treated with $[Rp\text{-}MP/DE]_6[PS]_5[Rp\text{-}MP/DE]_9$ oligomer 3678 [SEQ. ID. NO. 99]. CaSki cells grown in monolayers to about 50% confluency were transfected with 0, 0.3, 1 or 3 $\mu$M of oligomer 3678, or with 3 $\mu$M of control oligomer 3268 [SEQ. ID. NO. 102] using Lipofectamine™ (BRL). Sixteen hours after the transfection, intracellular RNA was extracted and RNase protection assays were carried out using an HPV-16 E7 $^{32}$P-labeled RNA probe. The amount of protected E7 or actin mRNA was quantified using a phosphoimager and the percentage of E7 or actin mRNA respect to Lipofectamine-treated controls was calculated.

Figure 11 depicts reduction of E7 mRNA levels in HPV-transformed CaSki cells treated with [Rp-MP/DE][PS]$_5$[Rp-MP/DE] oligomers 3678 [SEQ. ID. NO. 99], 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101], all

them targeted to the translation initiation codon of HPV-16 E7 and with control oligomer 3268 [SEQ. ID. NO. 102]. CaSki cells grown in monolayers to about 50% confluency were treated with 1 μM of oligomers and Lipofectamine™ (BRL) for sixteen hours. After the transfection, the cells were washed with CaSki cell culture medium and incubated with the same medium under tissue culture conditions for eight hours. Treatments were repeated for three consecutive days. Intracellular RNA was extracted and RNase protection assays were carried out using an HPV-16 E7 $^{32}$P-labeled RNA probe or a GAPDH $^{32}$P-labeled RNA probe. The amount of protected E7 or GAPDH mRNA was quantified using a phosphoimager and the percentage of E7 of GAPDH mRNA respect to Lipofectamine treated controls was calculated.

Detailed Description of the Invention

Preferred Oligomer Target Regions

Preferred target regions include the portion of an mRNA or pre-mRNA which includes the translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a polyadenylation signal, a 3'-untranslated region and a 5'-untranslated region. Preferably the mRNA or pre-mRNA codes for a human papilloma virus gene selected from E1, E2, E6 and E7. Preferred target sites include the splice donor at 847 in HPV-6b and HPV-11. A corresponding splice donor site is found in other HPVs, for example, at 880 in HPV-16, at 929 in HPV-18, at 877 in HPV-31, at 894 in HPV-33, at 982 in HPV-5, at 966 in HPV-33, at 982 in HPV-5, at 966 in HPV-8 and at 827 in HPV-1. Figure 8 depicts coding potentials of HPV-11 E-region transcripts and may be used to select appropriate target regions. Corresponding target regions for other HPVs may conveniently be determined by reference to alignment of HPV sequences such as that set forth in Figure 2A.

Preferred target regions include those from about -20 to about +20 nucleosides of a splice donor site or a splice acceptor site, a polyadenylation signal, or within a 3'-untranslated region or a 5'-untranslated region or that about -25 to +35 nucleotides of initiation codon.

According to an especially preferred aspect, provided are antisense oligomers which are complementary to a target region of an mRNA or pre-mRNA of an HPV which have from about 14 to about 35 nucleosidyl units, preferably from about 18 to about 24 nucleosidyl units, and more preferably from about 20 to 22 nucleosidyl units.

Especially preferred are oligomers complementary to a portion of the mRNA or pre-mRNA encoding the E7 gene, more preferably the target region is in the region of about -25 to about +35 relative to the initiation codon at +1 to +3. We believe that under physiological conditions a portion of the E6/E7 message forms a pseudoknot structure in a portion of the sequence 5' to the E7 initiation codon (*see* Figures 2A and 2B and Example E). The formation of the pseudoknot structure may be necessary for termination of E6 translation and/or for initiation of E7 protein translation from the polycistronic message (*see* Figure 1), since a frame-shift event would be necessary. Pseudoknots have been identified in other eukaryotic mRNAs and have been involved in frame-shift events (Draper, D.E., et al., *Current Opinions in Cell Biology*, Vol. 2, pp. 1099-1103 (1990)). The presence of the pseudoknot structure upstream from the translation initiation codon of E7 may reduce the accessibility of these sequences to targeting by antisense oligonucleotides (*see* Table I). Accordingly, according to a preferred aspect, provided are oligomers complementary to a portion of the E7 mRNA immediately 5'- or

immediately 3' to the pseudoknot region, more preferably immediately 3'- to the pseudoknot region.

Preferred Oligomers

Although the antisense oligomers of the present invention may incorporate any of the variety of internucleosidyl linkages or backbones, in certain instances preferred are oligomers have an RNase H-activating region and a non-RNase H-activating region. Preferably, the RNase H-activating region comprises a segment of at least three consecutive 2'-unsubstituted nucleosides linked by charged internucleosidyl linkages. Preferred charged internucleosidyl linkages include phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages. According to one preferred aspect, a mixed charged linkage sequence is used which includes at least two different charged nucleosidyl linkages. The non-RNase H-activating region comprises a segment of at least two linked nucleosidyl units linked by internucleosidyl linkages which do not activate (or serve as a substrate for) RNase H. According to an especially preferred aspect, at least one of the linkages in the non-RNase H-activating region is chirally-selected. Oligomers having an RNase H-activating region are further described in the commonly-assigned and copending United States Patent Application of Lyle J. Arnold, Jr., Mark A. Reynolds and Cristina Giachetti, "Chimeric Oligonucleoside Compounds", Serial No. 08/233,778, filed April 26, 1994, 08/238,177, filed May 4, 1994 and PCT/US94/13387, filed November 16, 1994. The disclosures of these applications are incorporated herein by reference.

The Oligomers provided herein may form a high affinity complex with a target sequence such as a nucleic acid with a high degree of selectivity. In addition, derivatized Oligomers may be used to bind with and then irreversibly modify a target site in a nucleic

acid by cross-linking (psoralens) or cleaving one or both strands (EDTA). By careful selection of a target site for cleavage, one of the strands may be used as a molecular scissors to specifically cleave a selected nucleic acid sequence. Alternatively, the Oligomers of the present invention may include an RNase H activating sequence.

According to one aspect of the present invention, these antisense Oligomers have a sequence which is complementary to a portion of the RNA transcribed from a selected target gene. Although the exact molecular mechanism of inhibition has not been conclusively determined, it has been suggested to result from formation of duplexes between the antisense Oligomer and the RNA transcribed from the target gene. The duplexes so formed may inhibit translation, processing or transport of an mRNA sequence.

According to an alternate aspect of the present invention, interference with or prevention of expression or translation of a selected RNA target sequence may be accomplished by triple helix formation using Oligomers of the present invention as a Triplex Oligomer Pair having sequences selected such that the Oligomers are complementary to and form a triple helix complex with the RNA target sequence and thereby interfere with or prevent expression of the targeted nucleic acid sequence. Such triple strand formation can occur in one of several ways. Basically, two separate or connected Oligomers may form a triple strand with the single stranded RNA. Further descriptions of the use of Oligomers (including Triplex Oligomer Pairs) to prevent or interfere with the expression of a target sequence of double or single stranded nucleic acid by formation of triple helix complexes is described in the copending U.S Patent Applications Serial Nos. 07/388,027, 07/751,813,

07/772,081 and 07/987,746, the disclosures of which are incorporated herein by reference.

As a general matter, the Oligomers employed will have a sequence that is complementary to the sequence of the target nucleic acid. However, absolute complementarity may not be required; in general, any Oligomer having sufficient complementarity to form a stable duplex (or triple helix complex as the case may be) with the target nucleic acid is considered to be suitable. Since stable duplex formation depends on the sequence and length of the hybridizing Oligomer and the degree of complementarity between the antisense Oligomer and the target sequence, the system can tolerate less fidelity (complementarity) when longer Oligomers are used. This is also true with Oligomers which form triple helix complexes. However, Oligomers of about 8 to about 40 nucleosidyl units in length which have sufficient complementarity to form a duplex or triple helix structure having a melting temperature of greater than about 40°C under physiological conditions are particularly suitable for use according to the methods of the present invention.

With respect to single stranded target sequences, we have found that two strands of a methylphosphonate Oligomer having methylphosphonate linkages (Second and Third Strands) and one strand of a complementary synthetic RNA Oligomer (First Strand) may form a triple helix complex. According to those experiments, the two methylphosphonate strands bind in a parallel orientation. Experiments described triple helix formation with methylphosphonate Oligomers of random sequence of A and G nucleosides which would not make triple helix complexes according to any of the "classical" triplet motifs.

These triple helix complexes formed by binding a target single stranded RNA and two methylphosphonate

Oligomers show high affinity (Tm > 50°C). Formation of these triple helix complexes has been shown to dramatically inhibit translation at sub-micromolar concentrations.

The triple helix complexes can be formed using Oligomers containing naturally occurring bases (i.e., A, C, G, T or U). Alternatively, if desired for increased stability, certain stabilizing bases such as 2-amino A (for A) or 5-methyl C may be used in place of the corresponding naturally occurring base. These bases may increase stability of the triple helix complex by having increased hydrogen bonding interactions and stacking interactions with other bases. Increased stability may result in increased affinity constants which increase potency.

The Oligomers provided herein may be derivatized to incorporate a nucleic acid reacting or modifying group which can be caused to react with a nucleic acid segment or a target sequence thereof to irreversibly modify, degrade or destroy the nucleic acid and thus irreversibly inhibit its functions.

Utility and Administration

These Oligomers may be used to inactivate or inhibit or alter expression of a particular gene or target sequence of the HPV in a living cell, allowing selective inactivation or inhibition or alteration of expression. The target sequence may be DNA or RNA, such as a pre-mRNA or an mRNA. mRNA target sequences include an initiation codon region, a coding region, a polyadenylation region, an mRNA cap site or a splice junction. These Oligomers could also be used to permanently inactivate, turn off or destroy genes which produced defective or undesired products or if activated caused undesirable effects.

Since the Oligomers provided herein may form duplexes or triple helix complexes or other forms of stable association with transcribed regions of nucleic acids, these complexes are useful in "antisense" or triple strand therapy. "Antisense" therapy as used herein is a generic term which includes the use of specific binding Oligomers to inactivate undesirable DNA or RNA sequences in vitro or in vivo.

Diseases and other conditions such as those caused by infection which a HPV are characterized by the presence of undesired DNA or RNA, which may be in certain instances single stranded and in other instances in double stranded. These diseases and conditions can be treated using the principles of antisense therapy as is generally understood in the art. Antisense therapy includes targeting a specific DNA or RNA target sequence through complementarity or through any other specific binding means, in the case of the present invention by formation of duplexes or triple helix complexes.

The Oligomers for use in the instant invention may be administered singly, or combinations of Oligomers may be administered for adjacent or distant targets or for combined effects of antisense mechanisms with the foregoing general mechanisms.

In therapeutic applications, the Oligomers can be formulated for a variety of modes of administration, including oral, topical or localized administration. It may be beneficial to have pharmaceutical formulations containing acid resistant Oligomers that may come in contact with acid conditions during their manufacture or when such formulations may themselves be made acidic, to some extent, in order to more compatible with the conditions prevailing at the site of application, e.g., the acid mantle of the skin. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton,

PA, latest edition. The Oligomer active ingredient is generally combined with a carrier such as a diluent or excipient which may include fillers, extenders, binding, wetting agents, disintegrants, surface-active agents, erodible polymers or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, and capsules.

Certain of the Oligomers of the present invention may be particularly suited for oral administration which may require exposure of the drug to acidic conditions in the stomach for up to about 4 hours under conventional drug delivery conditions and for up to about 12 hours when delivered in a sustained release from. For treatment of certain conditions it may be advantageous to formulate these Oligomers in a sustained release form. U.S. Patent No. 4,839,177 to Colombo et al., the disclosure of which is incorporated herein by reference, describes certain preferred controlled-rate release systems. For oral administration, these Oligomers have 2'-O-alkyl nucleosidyl units; these Oligomers are formulated into conventional as well as delayed release oral administration forms such as capsules, tablets, and liquids.

The Oligomers having 2'-O-alkyl nucleosidyl units may be particularly suited for formulation in preparations for topical administration, since the skin has an acid mantle, formulations including these acid resistant Oligomers may prove advantageous. This also can be advantageous in light of the finding that neutral Oligomers will cross skin and mucous membranes as described in U.S. Patent Application Serial No. 07/707,879 which is incorporated by reference. Also it may be desirable to provide formulations which include

acidic media when using acid-resistant neutral Oligomers.

For topical administration, the Oligomers for use in the invention are formulated into ointments, salves, eye drops, gels, or creams, as is generally known in the art.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucusal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, as well as formulations suitable for administration by inhalation, or suppositories.

To assist in understanding the present invention, the following examples are included which describe the results of a series of experiments. The following examples relating to this invention should not, of course, be construed in specifically limiting the invention and such variations of the invention, now known or later developed, which would within the purview of one skilled in the art are considered to fall within the scope of the present invention as hereinafter claimed.

Examples

Example 1

Preparation of MP($R_p$)/DE and MP($R_p$)/MP Dimer Synthons

A. Preparation of a (CT) Dimer Having a Chirally Pure Methylphosphonate Internucleosidyl Linkage Using Solution Phase Chemistry

Into a 2 L roto-evaporator flask was placed 10.0 g (28 mM) of 3'-tert-butyldimethylsilyl thymidine and 26.1 g (35 mM) of 5'-dimethoxytrityl-$N^4$-isobutyryl-3'-methyl-N,N-diisopropylaminophosphoramidite-2'-deoxycytidine. The solids were dissolved in 500 ml of acetonitrile and evaporated to dryness under vacuum. This process was repeated with another 500 ml of acetonitrile and then the flask was released under argon and stoppered with a rubber septa.

This dry solid foam was then dissolved in 500 ml of acetonitrile ("ACN"), and with manual stirring, treated all at once with 404 ml tetrazole (180 mM, 0.45 M tetrazole in THF). Manual stirring is continued for 30 seconds and then the flask is allowed to stand for another 2.5 minutes, after which time the reaction mix is treated all at once with 275 ml of an oxidizer solution ($I_2$/$H_2O$/lutidine/THF; 25 g/2.5 ml/100 ml/900 ml). The solution was stirred manually and allowed to stand at room temperature for 15 minutes. The resulting dark amber solution was then treated with bisulfite (2 g/25 ml $H_2O$), which upon addition, turned the solution light amber as it reacted with the excess iodide. The reaction mix was then concentrated to a thick oil and taken up in ethyl acetate ("EtOAc") (500 ml) and washed with saturated sodium bicarbonate (2 X 250 ml) and $H_2O$ (2 x 250 ml). The organic phase was dried over $MgSO_4$, filtered and concentrated to a light colored solid foam, which upon further drying yielded 35 grams of crude dimer.

The crude dimer was run on HPLC (reverse phase, Waters C18 bondapak) with a program (ACNMETH) starting with 50% acetonitrile and 0.1 M triethylammonium acetate (TEAA, pH ~ 7.0) which increased to 100% acetonitrile over 20 minutes with a linear gradient. Two major peaks were resolved, one at 4.5 minutes, which is residual lutidine and the other at 14.5 minutes which is the mixture of $R_p$ and $S_p$ diastereomers. The ratio of $R_p$ and $S_p$ was determined quantitatively by taking a 5 mg aliquot of the crude product and dissolving it in 1.5 ml of acetonitrile along with 0.5 ml of tetrabutylammonium fluoride (TBAF, 1 M solution in THF). After standing at room temperature for 10 minutes the sample was run on HPLC. Two new peaks were observed at 6.5 and 7.1 minutes and the later eluting peak was gone. The first new peak, which is believed to be the $S_p$ diastereomer, represented 66% (2/1) of the normalized value for the two peaks. The crude product was also analyzed by the (normal phase silica plate) in 75/25 EtOAc/$CH_2Cl_2$ ("75/25") with 5% methanol added. The tlc showed two spots with Rf's of 0.45 and 0.64, respectively; the faster running product (believed to be the $R_p$ form) was less intense than the slower moving one.

The $R_p$ diastereomer was separated on normal phase silica using a methanol step gradient in 75/25 EtOAc/$CH_2Cl_2$. A 7.5 cm by 60 cm column, was loaded with 700 g of silica (first slurried in 2.5 L of neat 75/25 EtOAc/$CH_2Cl_2$). The crude dimer was then dissolved in 75 ml of 75/25 EtOAc/$CH_2Cl_2$ and loaded onto the column. The column was started with 1% methanol and increased to 2% and finally 3% where the $R_p$ dimer began to elute. The $R_p$ dimer eluted cleanly over several bed volumes while maintaining 3% methanol in the eluent. The $S_p$ dimer was eluted later with 30% methanol. The $R_p$ dimer yield was 11.0 grams, while the $S_p$ yield was 17.8 grams. HPLC analysis (ACNMETH) was performed on the $R_p$ dimer and one

peak was observed at 14.5 minutes. The tlc (75/25 EtOAc/$CH_2Cl_2$, 5% methanol) of this product, revealed a single spot product with an Rf of 0.55 which, upon treatment with 10% sulfuric acid in ethanol and heat, was both trityl and sugar positive.

The newly resolved $R_p$ dimer, 11.0 g (0.011 M) was dissolved in 110 ml of ACN and treated all at once at room temperature with 22 ml of TBAF (0.022 M, 1 M in THF). The reaction mixture was allowed to stand overnight at ambient temperature. The next morning the reaction was determined to be complete by tlc (75/25, EtOAc/$CH_2Cl_2$ with 10% methanol); no starting material was detected but a small amount of 5'-DMT-dT was observed, which runs considerably faster on normal phase silica than the 3'-OH of the dimer. The reaction mixture was concentrated on a rotary evaporator to a thick oil which was then dissolved in $CH_2Cl_2$ (200 ml) and washed with saturated sodium bicarbonate (2 x 100 ml) and $H_2O$ (2 x 100 ml). The organic phase was dried over $MgSO_4$, filtered, and concentrated to a light yellow solid foam, which was purified on 100 grams of silica (75/25, EtOAc/$CH_2Cl_2$ with 5% methanol). The 5'-DMT-dT was removed but an impurity at 13.5 minutes (HPLC, ACNMETH) was detected which was first believed to be unreacted starting material (t-BDMS on) but after additional treatment with TBAF this was found not to be the case. A second column, using 100 g of silica and the same eluent was run and smaller fractions were taken; the column was able to successfully separate the two spots. The pure CT-$R_p$ dimer fractions were pooled and concentrated to yield 5.5 grams of a nearly white solid foam.

B. Preparation of a Chirally Pure (CT) MP($R_p$)/DE Dimer Synthon

Into a 100 ml round bottom flask was placed 0.5 g (0.55 mMol) CT-3'-OH dimer (product of Example 1A) which was rendered anhydrous by 3 x 20 ml co-evaporations with pyridine. The flask was released from the vacuum system under argon gas and stoppered with a rubber septa. The compound was redissolved in 10 ml acetonitrile and 200 µl (1.4 mMol, 2.5 eq) TEA were added. To the resulting mixture at room temperature and with manual stirring, was added in one portion 200 µl (0.90 mmol, 1.6 eq.) 2'-cyanoethyl-N,N-diisopropylchlorophosphoramidite. The reaction mixture was allowed to sit at room temperature before being analyzed by reverse phase HPLC. The HPLC (Beckman System Gold, C18 bondapak, ACN method Solution A was 50/50 ACN/0.1 M TEAA in water, pH 7 and Solution B was ACN. A gradient of 0 to 100% Solution B was run at a rate of 1 ml/minute over 25 minutes) showed complete conversion of starting material and a crude purity of greater than 90 percent. The diastereomers of the phosphoramidite were not resolved. The reaction mixture was concentrated under vacuum to a light yell solid foam. The foam was purified immediately by chromatography on 20 g of normal flash grade silica equilibrated with 5/1/5 ethyl acetate/ acetonitrile/methylene chloride with 2% TEA to give 0.5 g (82% yield) of the above-identified product as an off-which solid foam having a purity of 99.3% as determined by HPLC.

C. Preparation of a Chirally Pure (CT) MP($R_p$)/MP Dimer Synthon

The CT-3'-OH dimer, 5.5 g (6 mM), prepared as described in part A above, was rendered anhydrous with two co-evaporations with pyridine. The resulting solid foam was released from the rotary evaporator with argon and stoppered with a rubber septa. The solid foam was

dissolved in 100 ml of 9/1, ACN/$CH_2Cl_2$, then treated with 1.7 ml triethylamine (TEA, 12 mM). With magnetic stirring, the reaction mix was treated dropwise at room temperature with 1.5 ml chloromethyl-N,N-diisopropylamino phosphine (Cl-MAP, 8 mM). The reaction was monitored on HPLC (ACNMETH) and after 1.5 hours was complete, showing two main products, one at 3.5 minutes which was pyridine and a second at 14.3 minutes which was the desired amidite.

The reaction mixture was concentrated on a rotary evaporator using a partial vacuum; the flask which contained the resulting light amber sludge was released under argon and capped. The crude product was immediately passed through a flash column containing 60 grams of silica (first equilibrated in 1/1/1 ACN/EtOAc/$CH_2Cl_2$ with 3% TEA). The product was eluted quickly with this eluent and all U.V. positive fractions were pooled and concentrated. The resulting solid foam was co-evaporated with ACN to remove any residual TEA, then dried overnight under full vacuum. The final product, an off white solid foam, weight 5.0 grams.

Example 2

Preparation of (CU) 2'-O-Methyl MP($R_p$)/2'-O-Methyl DE and 2'-O-Methyl MP($R_p$)/2'-O-Methyl MP Dimer Synthons

A. Preparation of 2'-O-Methyl C Monomer

A 5.0 g (8 mmol) portion of 2'-O methyl cytidine was rendered anhydrous with pyridine co-evaporations (3 X 25 ml) and then dissolved in 50 ml acetonitrile. The solution was treated with 1.65 ml triethylamine ("TEA") (12 mmol, 1.5 eq.) and cooled in an ice bath. The solution was then treated with dropwise addition of 1.65 ml chloromethyl-N,N-diisopropylamino phosphine ("Cl-MAP") over two minutes. The ice bath was removed and the reaction mixture stirred for two hours. The reaction mixture (reaction was determined to be complete

by HPLC) was concentrated to dryness. The residue was dissolved in 20 ml ethyl acetate/heptane (1:1) with 4% TEA, then loaded onto 40 g silica gel equilibrated with the same solvent system. All UV absorbing eluent from the column was collected and pooled, then concentrated to give 5.5 g of the above-identified product (yield about 90%).

B. Preparation of Silyl-Protected 2'-O-Methyl Uridine

Into a 250 ml round bottom flask was placed 5.0 g (9.0 mmol) 5'-DMT, 2'O-methyl uridine which was rendered anhydrous with dimethylformamide (DMF) co-evaporations (3 X 25 ml). The resulting dry foam was taken up in 50 ml DMF, then treated all at once with 2.4 g (35 mmol, 3.9 eq.) imidazole, followed by dropwise addition of 3.0 ml (12 mmol, 1.3 eq.) t-butyldiphenylsilyl chloride. The reaction mixture was stirred at room temperature overnight.

The progress of the reaction was checked by HPLC (ACN method (Solution A was 50/50 ACN/0.1 M TEAA in water, pH 7 and Solution B was ACN; a gradient of 0 to 100% Solution B was run at a rate of 1 ml/minute over 25 minutes) and thin layer chromatography ("TLC") using 5% methanol in methylene chloride, and determined to be complete (no starting material was evident). The reaction mixture was then poured into ice water and taken up in methylene chloride, then washed several times with aqueous sodium bicarbonate and water. The organic phase was dried over magnesium sulfate, filtered and then concentrated to give 7.2 g of a solid foam which gave a single spot on TLC. The solid foam was then dissolved in 70 ml methylene chloride and treated (with rapid magnetic stirring) all at once with 70 ml benzene sulfonic acid, 2% by weight in 2:1 methylene chloride/methanol. After stirring for 15 minutes at room temperature, the reaction mixture was quenched with

10 ml TEA. The resulting detritylated compound was stripped down to a thick amber oil which was then loaded onto 150 g. silica gel equilibrated in heat methylene chloride. The product was eluted from the column using 2% methanol (in methylene chloride). After drying, 3.51 g of the above identified product were obtained (yield about 80%).

C. Preparation of (CU) 2'-O-Methyl MP($R_p$)/2'-O-Methyl DE Dimer

The silyl-protected 2'-O-methyl uridine monomer (product of Example 2B) (3.0 g, 6 mmol) was taken up in 30 ml anhydrous ACN. The 2'-O methyl cytidine amidite monomer (product of Example 2A) (5.5g, 7 mmol, 1.2 eq.) separately, was taken up in 55 ml ACN. Both solutions were allowed to stand over 3 Å molecular sieves overnight at room temperature.

The two solutions were carefully decanted into a single flask and treated with 94 ml tetrazole (0.45 M in ACN, 42 mmol, 7 eq). The resulting mixture was stirred for 4 minutes and then oxidized by addition of 1.5 ml (1.2 eq.) cumene hydroperoxide. The reaction mixture was concentrated to dryness, then taken up in methylene chloride and washed with aqueous sodium bicarbonate and water. The organic phase was dried over magnesium sulfate, filtered and concentrated to give 7.5 g. of a solid foam. The diastereomeric ratio as determined by HPLC by comparison of areas under peaks was 57/43 $S_p$ to $R_p$.

The $R_p$ diastereomer was isolated by column chromatography using two silica columns (100:1, silica to crude product, equilibrated in 3:1 ethylacetate/methyl chloride with an increasing methanol gradient from 1 to 5%). A total of 1.07 g of pure $R_p$ dimer was isolated.

D. Deprotection of (CU) 2'-O-Methyl Dimer

A 1.07 g (0.90 mmol) portion of the 2'-O methyl CU dimer (product of Example 2C) was dissolved in 10 ml THF and treated all at once with 1.5 ml (1 m in THF, 1.5 eq.) tetrabutylammonium fluoride ("TBAF"). The reaction mixture was stirred at room temperature of r 30 minutes after which time HPLC revealed complete deprotection of the silyl group had been achieved. The reaction mixture was concentrated and the concentrate purified on 10 g silica gel, eluting with 3:1 ethyl acetate/methylene chloride with 5% methanol. The clean fractions were concentrated to give 550 mg of the above-identified pure 5'-OH dimer.

E. Preparation of a Chirally Pure (CU) 2'-O-Methyl (MP/DE) Dimer Synthon

A 230 mg portion of 2'-O-methyl CU 3'-OH dimer (product of Example 2D) was rendered anhydrous by 2 X 5 ml co-evaporations in ACN. The resulting dry solid foam was dissolved in 2.5 ml ACN and then 73 $\mu$l (2.5 eq.) triethylamine ("TEA") and 94 $\mu$l (2.0 eq.) 2'-cyanoethyl-N,N-diisopropyl chlorophosphoramidite ($\beta$CNE) were added. The reaction mixture was stirred at room temperature for 2 hours at which time HPLC analysis determined the reaction to be complete. The reaction mixture was dissolved in eluent (3/1/1 ethylacetate/acetonitrile/methylene chloride with 4% TEA) and loaded onto 2 g silica gel equilibrated with 3/1/1 ethylacetate/acetonitrile/methylene chloride with 4% TEA. The column was run using 3/1/1 ethylacetate/acetonitrile/methylene chloride with 1% TEA. The clean fractions, 3 to 25, were concentrated, redissolved in acetonitrile and concentrated again to a solid foam. The foam was dried overnight under full vacuum to give 200 mg of the above-identified product.

F. Preparation of Chirally Pure (CU) 2'-O-Methyl MP($R_p$)/2'-O-Methyl MP Dimer Synthon

Into a 100 ml round bottom flask was placed 400 mg (0.372 mmole) of 2'-O methyl CU dimer (product of Example 2D); it was rendered anhydrous by 1 X 5 ml co-evaporation with acetonitrile. The dry foam was then released from the vacuum system under argon gas, dissolved in 4 ml ACN and stoppered with a rubber septa. The solution was treated with 2 equivalents TEA (103 $\mu$l, 0.744 mmol), followed by 1.75 equivalents chloro-methyl-N,N-diisopropyl phosphine ("Cl-MAP") (118 $\mu$l, 0.651 mmol). The reaction mixture was stirred for 1 hour at room temperature, after which time HPLC showed about 50/50 starting material/product. An additional 50 $\mu$l TEA and 70 $\mu$l Cl-MAP were then added and the mixture stirred for an hour. When HPLC showed only 80% conversion, an additional 30 $\mu$l TEA and 30 $\mu$l Cl-MAP were added and the resulting mixture stirred another hour. At this time HPLC revealed 6% starting material. The reaction mixture was concentrated to dryness. The residue was dissolved in 500 ml 3/1/3 ethylacetate/acetonitrile/methylene chloride with 4% TEA and loaded onto 5 g silica equilibrated in the same solvent system. Fractions were collected. The early fractions were contaminated with a yellow impurity and, thus, were pooled and concentrated separately. The product from those fractions was then repurified by chromatography using the same conditions and pooled with the clean product isolated from the first column. The combined products were co-evaporated with ACN (3 X 5 ml) and dried overnight under full vacuum to give 350 mg (77% yield) of the above identified product which HPLC showed to be 95.5% pure.

Example 3

Preparation of 2'-O-Methyl MPS($R_p$)/ 2'-O-Methyl-DE and 2'-O-Methyl MPS($R_p$)/ 2'-O-Methyl-MP Dimer Synthons

These dimer synthons are prepared by following the procedures described in Example 2, except that in Paragraph C, an equivalent amount of 3H-1,2-benzodithiole-3-one, 1,1-dioxide (Beaucage reagent) is substituted for cumene hydroperoxide. The procedures of Paragraphs 2E and 2F, respectively, lead to the phosphodiester and methylphosphothioate linkage combinations.

Example 4

Preparation of MPS($R_p$)/DE Dimer Synthons

These dimer synthons are prepared by following the procedures of Example 1, except in Paragraph A, an equivalent amount 3-H-1,2-benzodithiole-3-one, 1,1-dioxide (Beaucage reagent) is substituted for the oxidizer solution ($I_2$/$H_2O$/lutidine/THF).

Example 5

Preparation of MP($R_p$)/PS2 Dimer Synthons

The MP($R_p$)/PS2 dimer synthons are prepared as follows. Isometrically pure $R_p$ dinucleosides having a free 3'-OH are prepared according to the methods described in Example 1A. The dinucleoside is converted to the corresponding thiophosphoramidite using procedures such as those of Plotto et al. (Plotto et al, Tetrahedron 47:2449-61 (1991)) or Gorenstein et al., U.S. Patent No. 5,218,088. The dinucleoside is co-evaporated three times with anhydrous pyridine, followed by three co-evaporations with toluene. A portion of dinucleoside (10 mmoles) is dissolved in 200 ml anhydrous dichloromethane, then three equivalents of anhydrous diisopropylethylamine followed by 1.5 equivalents of chloro-N,N-diisopropylamino-

thiomethoxyphosphine are added at 0°C with stirring. The reaction is monitored by TLC until determined to be complete.

The product is worked up and purified using the procedures of Example 1B for isolation of the **MP($R_p$)/DE** phosphoramidite.

Example 6

Preparation of **MPS($R_p$)/PS2** Dimer Synthons

The **MPS($R_p$)/PS2** dimer synthons are prepared as follows. The isometrically pure $R_p$ dinucleoside with a free 3'-OH is prepared according to the methods of Example 4. Using the dinucleoside, the dimer synthon is prepared by the methods of Example 5.

Example 7

Preparation of **MPS($R_p$)**/2'-O Methyl **DE** Dimer Synthons

The **MPS($R_p$)**/2'-O-methyl **DE** dimer synthons are prepared using procedures analogous to those of Examples 1 and 3 but using the appropriate protected 2'-deoxynucleoside and protected 2'-O-methyl nucleosides.

Example 8

Preparation of a Poly-CT Oligomer Having Alternating **MP($R_p$)/DE** Internucleosidyl Linkages

An oligomer having the sequence 5'-(C*T)-(C*T)-(C*T)-(C*T)-(C*T)-(C*T)-(C*T)-A-3' was prepared using a C*T **MP($R_p$)/DE** dimer synthon prepared according to Example 1. The grouped dinucleosides indicate where the stereochemistry is fixed as the fast eluting isomer on silica gel (putative $R_p$) and the asterisks indicate the chirally pure linkages.

Manual couplings were used to synthesize the oligomer to conserve reagent, although the process can be done on an automated DNA synthesizer. The sequence

was synthesized from the 3'-terminus starting with methacrylate support bound deoxyadenosine.

The protected dinucleoside methylphosphonamidite (22 mg each per required coupling) freshly co-evaporated with pyridine and toluene to ensure dryness were placed into dried 1 ml glass autosampler vials and dissolved in anhydrous acetonitrile to a concentration of 0.1 M (200 μl per coupling). The vessels were purged with argon and tightly sealed with screw caps with teflon septa.

A 1 μmole scale DNA synthesis column (Milligen) was filled with 1 μmole of methacrylate support bound deoxyadenosine. The column was attached to a ring stand in a vertical orientation. A male-male luer fitting was attached to the bottom along with an 18 gauge needle to control the effluent. The column was washed with 10 ml acetonitrile using a syringe. The support bound nucleoside was detritylated by passing 3 ml of 2% dichloroacetic acid in dichloromethane through the column over 1.5 minutes. The orange, dimethoxytrityl cation bearing solution was reserved. The column was washed twice with 10 ml each of anhydrous acetonitrile.

The first coupling was accomplished as follows: 10 ml more anhydrous acetonitrile was passed through the column. Then, 200 μl of the CT methylphosphonamidite was drawn into a 1 ml syringe. Next, 200 μl of 0.45 M tetrazole in anhydrous acetonitrile was likewise drawn into the syringe containing the methylphosphonamidite. The reagents were rapidly mixed in the syringe, then slowly passed through the column dropwise over three minutes, being sure to lightly draw the plunger up and down to ensure adequate mixing with the support. After 3 minutes, 1 ml of the oxidizing reagent (0.1 M $I_2$ in 73% tetrahydrofuran, 25% 2,6-lutidine and 2% water) was passed through the column over one minute. The column was washed with 20 ml acetonitrile and then treated with 600 μl of a solution containing 20% (v/v) acetic

anhydride, 30% (v/v) acetonitrile, 50% (v/v) pyridine and 0.312% (w/v) dimethylaminopyridine. The column was then washed with 20 ml acetonitrile.

The above-described synthetic cycle was repeated until the synthesis was completed. The overall coupling efficiency based on dimethoxytrityl absorbance was 95.7%, for an average of 99.3% per coupling.

The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/$NH_4OH$ (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction allowed to sit for 6 hours at ambient temperature in order to go to completion. The supernatant containing the oligomer was then removed from the support and the support was rinsed twice with 2 ml of 1/1 acetonitrile/water; the washings were combined with the supernatant. The combined solution was diluted to 30 ml total volume with water and neutralized with approximately 4 ml of 6 N HCL. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 10 ml acetonitrile, 10 ml of 50% acetonitrile/100 mM triethylammonium bicarbonate, and 10 ml of 25 mM triethylammonium bicarbonate, sequentially. After the reaction solution was passed through the column, it was washed with 30 ml of water. The product was then eluted with 5 ml of 1/1 acetonitrile/water.

The oligomer was purified on HPLC using a Beckman Ultrasphere-reverse phase 4.5 X 250 mm column with an increasing gradient of acetonitrile in 0.5 M triethylammonium acetate (0% to 40% over 40 minutes). The isolated yield was 41 $OD_{260}$ units (35%). The

compound was characterized by electron spray mass spectrometry (calc. 4391/found 4391).

Alternatively, the above-identified oligomer can be synthesized on an automated DNA synthesizer. In this case the appropriate dimer synthons (as used above in the manual synthesis) are dissolved in acetonitrile to a concentration of 0.1 M as described above. The amidite solutions are placed in conical vessels on a Millipore Expedite DNA Synthesizer. All other reagents (oxidizer, deblock, capping reagents and activator) are prepared as described above for the manual synthesis, and applied to the appropriate positions on the instrument as instructed in the manual. Programming parameters for one synthesis cycle are as given in U.S. Patent Application Serial No. 08/158,014. The deprotection and purification of the oligomer is carried out as described above for the manually synthesized oligomer.

Example 9

Preparation of a Poly-CU Oligomer Having Alternating 2'-O-Methyl **MP($R_p$)**/2'-O-Methyl **DE** and 2'-O-Methyl **MP($R_p$)**/2'-O-Methyl **DE** Internucleosidyl Linkages

An oligomer having the sequence 5'(C*U)-(C*U)-(C*U)-(C*U)-(C*U)-(C*U)-(C*U)-A-3' was prepared using 2'-O-methyl **MP($R_p$)**/2'-O-methyl **DE** dimer synthons prepared according to Example 2 hereinabove.

The appropriate dimer synthons were dissolved in acetonitrile to a concentration of 0.1 M. All other reagents used were as described in Example 8.

A 1 μmole scale DNA synthesis column (Millipore) was filled with 1 μmole of methacrylate support bound deoxyadenosine. The dimer synthons were coupled sequentially from the 3'-terminus as described in Example 8 except that the coupling time was extended to two minutes. The overall coupling efficiency based on dimethoxytrityl absorbance was 50%, for an average of

91% per coupling. The dimethoxytrityl group was removed from the oligomer at the end of the synthesis.

The deprotection was carried out as described in Example 8. The crude yield was 103 $OD_{260}$ units. The oligomer was purified on HPLC with a Beckman Ultrasphere-$R_p$ using an increasing gradient of acetonitrile in 0.5 M triethylammonium acetate (10% to 30% over 30 minutes). The isolated yield was 39 $OD_{260}$ units (38%). The compound was characterized by electron spray mass spectrometry (calc. 4713/found 4712).

This oligomer can also be synthesized on an automated DNA synthesizer as follows. The appropriate dimer synthons (as used above in the manual synthesis are dissolved in acetonitrile as described in Example 8. The amidite solutions are placed in conical vessels on the Millipore Expedite DNA synthesizer. All other reagents (oxidizer, deblock, capping reagents and activator) are prepared as described in Example 8, and are applied to the appropriate positions on the instrument as instructed by the manual. The same coupling program as described in Example 8 is used except that the coupling time is extended to 2 minutes.

The deprotection is carried out as described in Example 8. The oligomer can be purified on HPLC using as described above for the manual synthesis.

Using similar procedures as described in detail in Example 8 of U.S. Patent Application Serial No. 08/154,013, the oligomer 5'-(C*U)-(C*U)-(C*U)-(C*U)-(C*U)-(C*U)-(C*U)-A-3' having 2'-O-methyl **MP**($R_p$)/2'-O-methyl **MP** (racemic) mixed linkages was prepared. The product was also characterized by electron spray mass spectroscopy (calc. 4699.5/found 4701). Automated synthesis may also be employed as explained above.

Example 10

Preparation of 5'-(T*A)-(G*C)-(T*T)-(C*C)-(T*T)-(A*G)-(C*T)-(C*C)-(T*G)-C-3' Having Repeated MP($R_p$)/MP Linkage Structures

The grouped dinucleosides indicate coupled dimers and the asterisk indicates where the stereochemistry is fixed (chirally defined or chirally pure) as the fast eluting isomer on silica gel (identified as $R_p$).

An oligomer having this sequence was synthesized using the appropriate protected dinucleotide methylphosphonamidites prepared using methods such as those described in Examples 1A and 1C above. Manual couplings were used to synthesize the oligomer to conserve reagent, although the process can be done on an automated DNA synthesizer from the 3' terminus starting with support-bound cytidine.

Each of the desired protected dinucleotide methylphosphonamidites (22 mg each per required coupling), T*A, G*C, T*T (2x), C*C (2x), A*G, C*T, and T*G, freshly co-evaporated with pyridine and toluene to ensure dryness, was placed into a dried 1 ml glass autosampler vial and dissolved with anhydrous acetonitrile to give a concentration of 0.1 M (200 μl were used per coupling). The vials were purged with argon and tightly sealed with screw caps with teflon septa.

A 1 μmole scale Milligen DNA synthesis column was filled with 1 μmole of support bound cytidine. The column was attached to a ring stand in a vertical orientation. A male-male leur fitting was attached to the bottom along with an 18 gauge needle to control the effluent. The column was washed with 10 ml of ACN using a syringe. The support bound nucleoside was then detritylated by passing 3 ml of 2% dichloroacetic acid in dichloromethane through the column over 1.5 minutes. The orange, dimethoxytrityl cation bearing solution was

reserved. The column was washed twice with 10 ml each of ACN (anhydrous).

The first coupling was accomplished by passing 10 ml more ACN (anhydrous) through the column. Then, 200 μl of the TG methylphosphonamidite was drawn into a 1 ml syringe. Next, 200 μL of 0.45 M tetrazole in anhydrous ACN was likewise drawn into the syringe containing the methylphosphonamidite. The reagents were rapidly mixed in the syringe, then slowly passed through the column dropwise over 3 minutes, being sure to lightly draw the plunger up and down to ensure adequate mixing with the support. After 3 minutes, 1 ml of the oxidizing reagent (0.1 M $I_2$ in 74.25% THF, 25% 2,6-lutidine, and 0.25% water) as passed through the column over 1 minute. The column was then washed with 20 ml of ACN. The column was then treated for 1 minute with 600 μl of a solution containing 20% (v/v) acetic anhydride, 30% (v/v) ACN, 50% (v/v) pyridine, and 0.312% (w/v) dimethyaminopyridine. The column was washed with 20 ml of ACN.

The synthetic cycle was then repeated with each dinucleotide methylphosphonamidite until the synthesis was completed. The order of addition of dimers after the initial T*G coupling was C*C, C*T, A*G, T*T, C*C, T*T, G*C, and T*A.

The dimethoxytrityl group was removed from the oligomer at the end of the synthesis.

The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/$NH_4OH$ (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction mixture allowed to sit for 6 hours at ambient temperature in order to go to completion. The

supernatant containing the oligomer was then removed from the support and the support was rinsed twice with 1 ml of 1/1 acetonitrile/water; the washings were combined with the supernatant. The combined solution was diluted to 50 ml total volume with water and neutralized with approximately 1.7 ml of glacial acetic acid. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 5 ml acetonitrile, 5 ml of 50% acetonitrile/water, and 5 ml of water, sequentially. After the reaction solution was passed through the column, it was washed with 50 ml of water. The product was then eluted with 2 ml of 1/1 acetonitrile/water.

The oligomer was purified by HPLC on a reverse phase column (Poros II R/H 4.6 x 100 mm) using a gradient of acetonitrile in water.

Coupling efficiencies are set forth in the table below.

Coupling Efficiencies of
Dinucleotide Methylphosphonamidites

| Dinucleotide | Coupling Efficiency |
|---|---|
| T*G | 99.7% |
| C*C | 90.2% |
| C*T | 91.8% |
| A*G | 85.5% |
| T*T | 97.8% |
| C*C | 83.6% |
| T*T | 100% |
| G*C | 86.2% |
| T*A | 92.4% |

Example 11

Preparation of 5'-(G*T)-(C*T)-(T*C)-(C*A)-(T*G)-(C*A)-(T*G)-(T*T)-(G*T)-C-3' Having Repeated MP($R_p$)/MP Linkage Structures

The grouped dinucleotides indicate coupled dimers and the asterisk indicates where the stereochemistry is fixed.

This sequence was synthesized using the appropriate protected $R_p$ dinucleotide methylphosphonamidites prepared and isolated using procedures such as those described in Examples 1A and 1C above. Manual couplings were used to synthesize the oligomer in order to conserve reagent. However, if desired, the process can be done on an automated DNA synthesizer from the 3' terminus starting with methacrylate support bound 2'-deoxycytidine.

Each of the desired protected dinucleotide methylphosphonamidites (100 mg), G*T, T*T, T*G, C*A, T*G, C*A, T*C, C*T, and G*T was placed into a dried 3 ml glass conical vial and dissolved with anhydrous acetonitrile to a concentration of 0.1 M. Molecular sieves (3 Å) (0.5 ml volume) were added to each vessel, the vessels purged with argon, and tightly sealed with screw caps with teflon septa. The reagents were allowed to stand overnight prior to use.

A 1 μmole scale Milligen DNA synthesis column was filled with 1 μmole of methacrylate support bound 2'-deoxycytidine. The column was attached to a ring stand in a vertical orientation. A male-male luer fitting was attached to the bottom along with an 18 gauge needle to control the effluent. The column was washed with 10 ml of ACN using a syringe. The support bound nucleoside was then detritylated by passing 3 ml of 2.5% dichloroacetic acid in dichloromethane through the column over 3.0 minutes. The orange, dimethoxytrityl cation bearing solution was reserved. The column was washed twice with 10 ml each of ACN (anhydrous).

The first coupling was accomplished by passing 10 ml more ACN (anhydrous) through the column. Then 200 μl of the G*T methylphosphoramidite was drawn into a 1 ml syringe. Next, 200 μl of 0.45 M tetrazole in anhydrous ACN was likewise drawn into the syringe containing the methylphosphonamidite. The reagents were rapidly mixed in the syringe, then slowly passed through the column dropwise over 1 minute, being sure to lightly draw the plunger up and down to ensure adequate mixing with the support. After 3 minutes, 1 ml of the oxidizing reagent (0.1 M $I_2$ in 74.25% THF, 25% 2,6-lutidine, and 0.25% water) was passed through the column over 1 minute. The column was then washed with 20 ml of ACN. The column was then treated for 1 minute with 600 μl of a solution containing 20% (v/v) acetic anhydride, 30% (v/v) ACN, 50% (v/v) pyridine, and 0.312% (w/v) dimethyaminopyridine. The column was washed with 20 ml of ACN.

The synthetic cycle was then repeated with each dinucleotide methylphosphonamidite until the synthesis was completed. The order of addition of dimers after the initial G*T coupling was T*T, T*G, C*A, T*G, C*A, T*C, C*T and G*T.

The dimethoxytrityl group was removed from the oligomer at the end of the synthesis.

The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/$NH_4OH$ (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction allowed 6 hours to go to completion. The supernatant containing the oligomer was then removed from the support and the support was rinsed twice with 1 ml of 1/1 acetonitrile/water; the washings were combined

with the supernatant. The combined solution was diluted to 30 ml total volume with water and neutralized with approximately 1.7 ml of glacial acetic acid. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 5 ml acetonitrile, 5 ml of 50% acetonitrile/water, and 5 ml of water, sequentially. After the reaction solution was passed through the column it was washed with 5 ml of water. The product was then eluted with 2 ml of 1/1 acetonitrile/water.

The oligomer was purified by HPLC on a reverse phase column (Poros II R/H 4.6 x 100 mm) using a gradient of acetonitrile in water.

Example 12

Preparation of 5'-(G*A)-(G*G)-(A*G)-(G*A)-(G*G)-(A*G)-(G*A)-(A*G)-G-3' Having Repeated MP($R_p$)/MP Linkage Structures

The grouped dinucleosides indicate the coupled dimers and the asterisks indicates where the stereochemistry is fixed (chirally defined or chirally pure) as the fast eluting dimer isomer on silica gel (identified as $R_p$).

This oligomer was prepared using automated synthesis coupling G*A, G*G and A*G MP($R_p$)/MP dimer synthons prepared according to the procedures of Examples 1A and 1C.

An amount of G*A, G*G and A*G dimer synthons was dissolved in acetonitrile to give a concentration of 0.1 M and stored over 3 Å molecular sieves (Millipore, Milford, MA) overnight.

The dissolved dimers, with molecular sieves, were placed in conical vessels on a Millipore Expedite DNA Synthesizer which as equipped with end-line filters to remove particulates. All other reagents (oxidizer, deblock, capping reagents and activator) were prepared

and applied to the appropriate positions on the instrument as instructed in the manual. The coupling program was modified to place the oxidizing step immediately subsequent to the coupling step in order to reduce backbone cleavage prior to oxidation. (See Hogrefe, R.I., et al. "An Improved Method for the Synthesis and Deprotection of Methylphosphonate Oligonucleotides" in Methods in Molecular Biology, vol. 20: Protocols for Oligonucleotides and Analogs (ed. Agrawal, S.) pages 143-164, Humana Press, Totowa N.Y. (1983). The programming parameters for one synthesis cycle ("Syn4all-1 μmol") are set forth in I of U.S. Patent Application Serial No. 08/154,013.

A 1 μmole scale DNA synthesis column (Millipore) was filled with 1 μmol of methacrylate support-bound deoxyguanosine and was placed on the DNA synthesizer. The dimers were coupled sequentially from the 3′ terminus. The dimethoxytrityl protecting group was removed from the oligomer at the end of the synthesis.

The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/$NH_4OH$ (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction allowed 6 hours to go to completion. The supernatant containing the oligomer was then removed from the support and the support rinsed twice with 1 ml of 1/1 acetonitrile/water, when combined with the supernatant. The combined solution was diluted to 50 ml total volume with water and neutralized with approximately 1.7 ml of glacial acetic acid. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 5 ml acetonitrile, 5 ml of 50% acetonitrile/water, and 5 ml

of water, sequentially. After the reaction solution was passed through the column, it was washed with 5 ml of water. The product was then eluted with 1.8 ml of 1/1 acetonitrile/water.

The crude yield was 87 $OD_{260}$ units. The Oligomers was purified on HPLC using a $\beta$-cyclobond standard phase 4.5 X 250 mm column (Azetec, Inc. Whippany, NJ) with a decreasing gradient (80% to 40%) of acetonitrile in 0.05 M triethylammonium acetate (pH 7). The isolated yield was 22 $OD_{260}$ units (25%). The product was characterized by electron spray mass spectrometry (calc. 5407/found 5401).

Example 13

Preparation of an Oligomer Having Alternating MP($R_p$)/PS Internucleosidyl Linkages

An oligomer having alternating MP($R_p$)/PS internucleosidyl linkages is prepared using dimer synthons. All the parameters of the synthesis, deprotection and purification are as described in Example 8, except that the oxidizing reagent is replaced by a 0.1 M solution of 3H-1,2-benzodithiole-3-one, 1,1-dioxide or a 0.1 M solution of sulfur in 1/1 carbon disulfide/diisopropylethylamine.

Example 14

Preparation of an Oligomer Having Alternating MPS($R_p$)/DE Internucleosidyl Linkages

An oligomer having alternating MPS($R_p$)/DE internucleosidyl linkages is prepared using the dimer synthons of Example 4. All other parameters of synthesis, deprotection and purification are as described in Example 8.

Example 15

Preparation of an Oligomer Having Alternating MPS($R_p$)/PS Internucleosidyl Linkages

An oligomer having alternating MPS($R_p$)/PS internucleosidyl linkages is prepared using the dimer synthons of Example 4. All of the parameters of synthesis, deprotection and purification are as described in Example 8, except that the oxidizing reagent is replaced by a 0.1 M solution of 3H-1,2-benzodithiole-3-one, 1,1-dioxide or a 0.1 M solution of sulfur in 1/1 carbon disulfide/diisopropylethylamine.

Example 16

Preparation of an Oligomer Having Alternating MP($R_p$)/PS2 Internucleosidyl Linkages

An oligomer having alternating MP($R_p$)/PS2 internucleosidyl linkages is prepared using the dimer synthons of Example 5. All of the parameters of synthesis, deprotection and purification are as described in Example 15.

Example 17

Preparation of an Oligomer Having Alternating MPS($R_p$)/PS2 Internucleosidyl Linkages

An oligomer having alternating MPS($R_p$)/PS2 internucleosidyl linkages is prepared using the dimer synthons of Example 6. All of the parameters of synthesis, deprotection and purification are as described in Example 16.

Example 17A

Preparation of an Oligomer Having Alternating MP($R_p$)/2'-O-Methyl DE Internucleosidyl Linkages

An oligomer having alternating MP($R_p$)/2'-O-Methyl DE internucleosidyl linkages is prepared using dimer synthons similar to those of Example 7. All other

parameters of synthesis, deprotection and purification are as described in Example 9.

Example 18

Preparation of an Oligomer Having Alternating MP($R_p$)/MPS Internucleosidyl Linkages

The preparation of an oligomer having alternating MP($R_p$)/MPS internucleosidyl linkages is accomplished using dimer synthons prepared according to Examples 1A and 1C and dissolved and stored over molecular sieves. The oxidizing reagent is a 0.1 M solution of 3H-1,2-benzodithiole-3-one, 1,1-dioxide ("Beaucage Reagent", See, Iyer, R.P. et al., JACS 112:1254-1255 (1990)) or a 0.1 M solution of sulfur in 1/1 carbon disulfide/diisopropylethylamine, with synthesis proceeding generally as described in Example 12.

Example 19

Preparation of an Oligomer Having 2'-O-Methyl Nucleosidyl Units and Alternating MP($R_p$)/MPS Internucleosidyl Linkages

This oligomer is prepared using the dimer synthons as described in Examples 2A-2D and 2F and following the general synthetic procedures of Example 8 of U.S. Patent Application Serial No. 08/154,013, except that the oxidizing reagent described therein is a 0.1M solution of 3H-1,2-benzodithiole-3-one, 1,1-dioxide or a 0.1 M solution on 1/1 carbon disulfide/diisopropylamine.

Example 20

Preparation of an Oligomer Having 2'-O-Methyl Nucleosidyl Units and Alternating MPS($R_p$)/MP Internucleosidyl Linkages

This oligomer is prepared using dimer synthons as described in Example 3 above and by following the

parameters of synthesis, deprotection and purification of Example 19.

Example 21

Preparation of an Oligomer Having Alternating MPS($R_p$)/MP Internucleosidyl Linkages

This oligomer is prepared using dimer synthons prepared according to Examples 1A and 1C, substituting Beaucage reagent for the oxidizer in Example 1A, and by following the parameters of synthesis, deprotection and purification as described above in Example 12.

Example 22

Preparation of an Oligomer Having Alternating MPS($R_p$)/MPS Internucleosidyl Linkages

This oligomer is prepared using dimer synthons as referred to in Example 21 and by following the parameters of synthesis, deprotection and purification as described above in Example 12, except that the oxidizing reagent used therein is replaced by a 0.1 M solution of 3H-1,2-benzodithiole, 1,1-dioxide or a 0.1 M solution of sulfur in 1/1 carbon disulfide/ diisopropylethylamine.

Example 23

Preparation of 2'-F Dimer Synthons

Dimer synthons useful in the preparation of the oligomers of the present invention may be prepared using 2'-fluoronucleosides. Methods for preparation of 2'-fluoronucleosides have been reported and are known to those skilled in the art. (See, e.g.: Codington, JOC Vol. 29 (1964) (2'-F U); Mangel, Angew. Chem. 96:557-558 (1978) and Doen, JOC 32:1462-1471 (1967) (2'-F C); Ikehara, Chem. Pharm. Bull. 29:1034-1038 (1981) (2'-F G); Ikehara, J. Carbohydrates, Nucleosides, Nucleotides

7:131-140 (1980) (2'-F A), and also Krug, A, Nucleosides & Nucleotides 8:1473-1483 (1989).

The preparation of dimer synthons using 2'-fluoronucleosides may be accomplishing using the procedures analogous to those described for the 2'-O-methyl dimer synthons (See, e.g., Examples 2, 3, and 7). The resulting dimer synthons may be used to prepare oligomers using methods analogous to the methods used for the 2'-O-methyl dimer synthons such as in Example 9.

Example 24

Preparation of MP($R_p$)/MP($R_p$)/DE and MP($R_p$)/MP($R_p$)/MP Trimer Synthons

The above-identified trimer synthons are prepared using the MP($R_p$)/MP dimer synthons of Example 1C. The dimer synthon is coupled to a 5'-hydroxy, 3'-silylated nucleoside according to the methods of Example 1A for the coupling of the 3'-nucleoside to the monomer phosphoramidite.

The selected 5'-hydroxy, 3'-silylated nucleoside (1 equivalent) and isomerically pure $R_p$ dimer methylphosphonamide (1.25 equivalents) are weighed into a round bottom flask and dried by co-evaporation with acetonitrile. The resulting foam is dissolved in acetonitrile and treated with a solution of 0.45 M tetrazole in acetonitrile (4.5 equivalents). After 3 minutes, the reaction mixture is oxidized and the reaction product is worked up as described in Example 1A. The diastereoisomers of the 3'-silylated trimer are resolved on a silica gel column as described in Example 1A for resolution of the dimer isomers. The configuration of the separated diastereoisomers is determined using 2-D nmr (ROSEY). The trimer having the desired chiral configuration ($R_p/R_p$) of the two internucleosidyl linkages is converted to a trimer synthon by reaction with chloro-$\beta$-cyanoethoxy-N,N-

diisopropylaminophosphoramidite using methods as described in Example 1B. The trimer synthon is worked up and purified using methods as described in Example 1B to achieve the MP($R_p$)/MP($R_p$)/DE trimer.

Using similar procedures, an MP($R_p$)/MP($R_p$)/MP phosphoramidite synthon may be obtained by using chloromethyl-N,N-diisopropylaminophosphine in the final reaction as described in Example 1C for the corresponding dimer synthon. Workup and purification are as described in Example 1C.

Example 25

Preparation of 2'-O-Allyl Dimer and Trimer Synthons and Their Use in Oligomer Synthesis

The dimer and trimer synthons described, for example, in Examples 1 and 24 can be prepared using 2'-O-allyl nucleosides. The preparation of 2'-O-allyl nucleosides and their use in the preparation of oligomers has been reported (see e.g. Iribarren, et al. (1990) Proc. Natl. Acad. Sci. (USA) 87:7747-51; and Lesnik et al. (1983), Biochemistry 32:7832-8), and such substituted nucleosides are commercially available. The nucleosides are used to prepare dimer and trimer synthons using procedures described hereinabove. The synthons are used to prepare oligomers using methods such as those described in Examples 10, 11, 12, 13 and others above.

Example 26

Preparation of an Oligomer Having MP($R_p$)/MP/DE Internucleosidyl Linkages

The above-identified oligomer is prepared using the trimer synthons of Example 24, or by those in Example 20 of U.S. Patent Application Serial No. 08/154,014, and by following the methods described in Example 8, substituting the trimer synthons for dimer synthons.

All other parameters of synthesis, deprotection and purification are as described in Example 8.

Example 27

Preparation of an Oligomer Having MP($R_p$)/MP($R_p$)/MP Internucleosidyl Linkages

The above-identified oligomer is prepared using the procedures described in Example 14 of U.S. Patent Application Serial No. 08/154,013.

Example 28

Preparation of Oligoribonucleosides

Oligoribonucleotides used in the present examples may be synthesized using general procedures such as described below.

The appropriate 5'-O-dimethoxytrityl-2'-O-tert-butyldimethylsilyl-3'-O-N,N-diisopropyl-β-cyanoethylphosphoramidite nucleosides (Millipore, Hilford, MA) were used for synthesis. Syntheses were done on a 1 μmole scale with a Milligen 8750 automated DNA synthesizer using standard Milligen phosphoramidite procedures with the exception that the coupling times were extended to 12 minutes to allow adequate time for the more sterically hindered 2'-O-tert-butyldimethylsilyl RNA monomers to react. The syntheses were begun on control-pore glass bound 2'-O-tert-butyldimethylsilyl ribonucleosides purchased from Millipore. All other oligonucleotide synthesis reagents were as described in Millipore's standard protocols.

After synthesis, the oligonucleotides were handled under sterile, RNase-free conditions. Water was sterilized by overnight treatment with 0.5% diethylpyrocarbonate followed by autoclaving. All glassware was baked for at least 4 hours at 300°C.

The oligonucleotides were deprotected and cleaved from the support by first treating the support bound

oligomer with 3/1 ammonium hydroxide/ethanol for 15 hours at 55°C. The supernatant, which contained the oligonucleotide, was then decanted and evaporated to dryness. The resultant residue was then treated with 0.6 mL of 1 M tetrabutylammonium fluoride in tetrahydrofuran (which contained 5% or less water) for 24 hours at room temperature. The reaction was quenched by the addition of 0.6 mL of aqueous 2 M triethylammonium acetate, pH 7. Desalting of the reaction mixture was accomplished by passing the solution through a Bio-Rad 10DG column using sterile water. The desalted oligonucleotide was then dried.

Purification of the oligoribonucleotides was carried out by polyacrylamide gel electrophoresis (PAGE) containing 15% 19/1 polyacrylamide/bis-acrylamide and 7 M urea using standard procedures (See Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, pages 184-185 (Cold Spring Harbor 1982)). The gels were 20 cm wide by 40 cm long and 6 mm in width. The oligoribonucleotides (60 OD Units) were dissolved in 200 μL of water containing 1.25% bromophenol blue and loaded onto the gel. The gels were run overnight at 300 V. The product bands were visualized by UV backshadowing and excised, and the product eluted with 0.5 M sodium acetate overnight. The product was desalted with a Waters C18 Sep-Pak cartridge using the manufacturer supplied protocol. The product was then $^{32}P$ labelled by kinasing and analyzed by PAGE.

Example 29

Preparation of Racemic Methylphosphonate Oligonucleotides

Various racemic oligomers were synthesized using 5'-(dimethoxytrityl) deoxynucleoside-3'-[(N,N-diisopropylamino)methyl]-phosphonoamidite monomers. Solid-phase synthesis was performed on methacrylate polymer supports

with a Biosearch Model 8750 DNA synthesizer according to the manufacturer's recommendations except for the following modifications: the monomers were dissolved in acetonitrile at a concentrations of 100 mM, except dG, which was dissolved in 1/1 acetonitrile/dichloromethane at 100 mM. DEBLOCK reagent = 2.5% dichloroacetic acid in dichloromethane. OXIDIZER reagent = 25 g/L iodine in 0.25% water, 25% 2,6-lutidine, 72.5% tetrahydrofuran. CAP A = 10% acetic anhydride in acetonitrile. CAP B = 0.625% N,N-dimethylaminopyridine in pyridine.

The dimethoxytrityl group was removed from the oligonucleotide at the end of the synthesis.

The oligonucleotide was then cleaved from the support and deprotected. The support bound oligonucleotide was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/$NH_4OH$ (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction allowed 6 hours to go to completion. The supernatant containing the oligonucleotide was then removed from the support and the support rinsed twice with 2 ml of 1/1 acetonitrile/water, when combined with the supernatant. The combined solution was diluted to 30 ml total volume with water and neutralized with approximately 4 ml of 6 N HCl. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 10 ml acetonitrile, 10 ml of 50% acetonitrile/100 mM triethylammonium bicarbonate, and 10 ml of 25 mM triethylammonium bicarbonate, sequentially. After the reaction solution was passed through the column it was washed with 30 ml of water. The product was then eluted with 5 ml of 1/1 acetonitrile/water.

The oligonucleotide was purified by HPLC on a reverse phase column (Whatman RAC II) using a gradient of acetonitrile in 50 mM triethylammonium acetate.

Example 30

Chimeric Oligonucleotide Assembly From **MP($R_p$)/MP** and **MP($R_p$)/DE** Dimer Synthons and Phosphoramidite and Methylphosphonamidite Monomer Synthons

**MP($R_p$)/MP** dimer synthons contained a methylphosphoramidite coupling group at the 3' end. When coupled together to make an oligomer, these synthons give racemic methylphosphonate linkages at every other position. $R_p$-MP/DE dimer synthons contained a β-cyanoethyl phosphoramidite coupling group at the 3'-end. Both types of dimer synthons were synthesized as described in Example 1. Methylphosphonamidite monomer synthons were synthesized at JBL Scientific (San Luis Obispo, CA). Betacyanoethyl phosphoramidite monomer synthons were purchased from Milligen/Biosearch.

All synthons were coupled using a Milligen Expedite™ automated DNA synthesizer. The coupling programs for each synthon were as tabulated below. To generate a phosphorothioate bond during a coupling step, the program "Thioate-5μM" was used with either a dimer or monomer synthon containing a β-cyanoethyl phosphoramidite coupling group.

## DIESTER -- 5 μM

```
/* ------------------------------------------------------------------------
/*       Function          Mode    Amount   Time(sec)       Description
/*                                 /Arg1    /Arg2
/* ------------------------------------------------------------------------
$Deblocking
  144 /* Advance Frac */  NA          1        0      "Event out ON"
    0 /* Default      */  WAIT        0      1.5      "Wait"
  141 /* Photometer S */  NA          1        1      "START data collection"
   16 /* Dblk         */  PULSE      10        0      "Dblk to column"
   16 /* Dblk         */  PULSE     200       49      "Deblock"
   38 /* Wsh A to Cl  */  PULSE      80        0      "Flush system with Wsh A"
  141 /* Photometer S */  NA          0        1      "STOP data collection"
   39 /* Gas A to Cl  */  PULSE      10        0      "Gas A to Cl waste"
  144 /* Advance Frac */  NA          2        0      "Event out OFF"
   12 /* Wsh A        */  PULSE     200        0      "Wsh A"
$Coupling
    1 /* Wsh          */  PULSE      10        0      "Flush system with Wsh"
    2 /* Act          */  PULSE      10        0      "Flush system with Act"
   18 /* A + Act      */  PULSE       5        0      "Monomer + Act to column"
   18 /* A + Act      */  PULSE      18       60      "Couple monomer"
    2 /* Act          */  PULSE       3       10      "Couple monomer"
    1 /* Wsh          */  PULSE       7     56.1      "Couple monomer"
    1 /* Wsh          */  PULSE      50        0      "Flush system with Wsh"
$Capping
   13 /* Caps         */  PULSE      25        0      "Caps to column"
   12 /* Wsh A        */  PULSE      50        0      "Wsh A"
   12 /* Wsh A        */  PULSE     150        0      "End of cycle wash"
$Oxidizing
   15 /* Ox           */  PULSE      50       30      "Ox"
   12 /* Wsh A        */  PULSE      50        0      "Flush system with Wsh A"
$Capping
   13 /* Caps         */  PULSE      25        0      "Caps to column"
   12 /* Wsh A        */  PULSE      50        0      "Wsh A"
   12 /* Wsh A        */  PULSE     150        0      "End of cycle wash"
```

## THIOATE -- 5 μM

```
/* ------------------------------------------------------------------------
/*       Function          Mode    Amount   Time(sec)       Description
/*                                  /Arg1    /Arg2
/* ------------------------------------------------------------------------
$Deblocking
  144 /* Advance Frac */  NA          1        0      "Event out ON"
    0 /* Default      */  WAIT        0      1.5      "Wait"
  141 /* Photometer S */  NA          1        1      "START data collection"
   16 /* Dblk         */  PULSE      10        0      "Dblk to column"
   16 /* Dblk         */  PULSE     200       49      "Deblock"
   38 /* Wsh A to Cl  */  PULSE      80        0      "Flush system with Wsh A"
  141 /* Photometer S */  NA          0        1      "STOP data collection"
   39 /* Gas A to Cl  */  PULSE      10        0      "Gas A to Cl waste"
  144 /* Advance Frac */  NA          2        0      "Event out OFF"
   12 /* Wsh A        */  PULSE     200        0      "Wsh A"
$Coupling
    1 /* Wsh          */  PULSE      10        0      "Flush system with Wsh"
    2 /* Act          */  PULSE      10        0      "Flush system with Act"
   23 /* 6 + Act      */  PULSE       6        0      "Monomer + Act to column"
   23 /* 6 + Act      */  PULSE      17       60      "Couple monomer"
    2 /* Act          */  PULSE       4       10      "Couple monomer"
    1 /* Wsh          */  PULSE       7     55.9      "Couple monomer"
    1 /* Wsh          */  PULSE      50        0      "Flush system with Wsh"
$Capping
   13 /* Caps         */  PULSE      25        0      "Caps to column"
   12 /* Wsh A        */  PULSE      50        0      "Wsh A"
   12 /* Wsh A        */  PULSE     150        0      "End of cycle wash"
$Oxidizing
   17 /* Aux          */  PULSE       5        0      "SOx"
   17 /* Aux          */  PULSE      45       60      "SOx"
   12 /* Wsh A        */  PULSE      50        0      "Flush system with Wsh A"
$Capping
   13 /* Caps         */  PULSE      25        0      "Caps to column"
   12 /* Wsh A        */  PULSE      50        0      "Wsh A"
   12 /* Wsh A        */  PULSE     150        0      "End of cycle wash"
```

## METHYLPHOSPHONATE -- 5 μM

| /* Function | Mode | Amount /Arg1 | Time(sec) /Arg2 | Description |
|---|---|---|---|---|
| SDeblocking | | | | |
| 144 /* Advance Frac */ | NA | 1 | 0 | "Event out ON" |
| 0 /* Default */ | WAIT | 0 | 1.5 | "Wait" |
| 141 /* Photometer S */ | NA | 1 | 1 | "START data collection" |
| 16 /* Dblk */ | PULSE | 10 | 0 | "Dblk to column" |
| 16 /* Dblk */ | PULSE | 200 | 49 | "Deblock" |
| 38 /* Wsh A to Cl */ | PULSE | 80 | 0 | "Flush system with Wsh A" |
| 141 /* Photometer S */ | NA | 0 | 1 | "STOP data collection" |
| 39 /* Gas A to Cl */ | PULSE | 10 | 0 | "Gas A to Cl waste" |
| 144 /* Advance Frac */ | NA | 2 | 0 | "Event out OFF" |
| 12 /* Wsh A */ | PULSE | 200 | 0 | "Wsh A" |
| SCoupling | | | | |
| 1 /* Wsh */ | PULSE | 10 | 0 | "Flush system with Wsh" |
| 2 /* Act */ | PULSE | 10 | 0 | "Flush system with Act" |
| 18 /* A + Act */ | PULSE | 5 | 0 | "Monomer + Act to column" |
| 18 /* A + Act */ | PULSE | 18 | 60 | "Couple monomer" |
| 2 /* Act */ | PULSE | 3 | 10 | "Couple monomer" |
| 1 /* Wsh */ | PULSE | 7 | 56.1 | "Couple monomer" |
| 1 /* Wsh */ | PULSE | 50 | 0 | "Flush system with Wsh" |
| SOxidizing | | | | |
| 15 /* Ox */ | PULSE | 50 | 30 | "Ox" |
| 12 /* Wsh A */ | PULSE | 50 | 0 | "Flush system with Wsh A" |
| SCapping | | | | |
| 13 /* Caps */ | PULSE | 25 | 0 | "Caps to column" |
| 12 /* Wsh A */ | PULSE | 50 | 0 | "Wsh A" |
| 12 /* Wsh A */ | PULSE | 150 | 0 | "End of cycle wash" |

MP($R_p$)/MP -- 5 μM

```
/* ------------------------------------------------------------------------
/*       Function              Mode    Amount   Time(sec)      Description
/*                                      /Arg1    /Arg2
/* ------------------------------------------------------------------------
$Deblocking
  144 /* Advance Frac */   NA          1        0      "Event out ON"
    0 /* Default      */   WAIT        0      1.5      "Wait"
  141 /* Photometer S */   NA          1        1      "START data collection"
   16 /* Dblk         */   PULSE      10        0      "Dblk to column"
   16 /* Dblk         */   PULSE     200       49      "Deblock"
   38 /* Wsh A to Cl  */   PULSE      80        0      "Flush system with Wsh A"
  141 /* Photometer S */   NA          0        1      "STOP data collection"
   39 /* Gas A to Cl  */   PULSE      10        0      "Gas A to Cl waste"
  144 /* Advance Frac */   NA          2        0      "Event out OFF"
   12 /* Wsh A        */   PULSE     200        0      "Wsh A"
$Coupling
    1 /* Wsh          */   PULSE      10        0      "Flush system with Wsh"
    2 /* Act          */   PULSE      10        0      "Flush system with Act"
   18 /* A + Act      */   PULSE       5        0      "Monomer + Act to column"
   18 /* A + Act      */   PULSE      18       60      "Couple monomer"
    2 /* Act          */   PULSE       3       10      "Couple monomer"
    1 /* Wsh          */   PULSE       7     56.1      "Couple monomer"
    1 /* Wsh          */   PULSE      50        0      "Flush system with Wsh"
$Oxidizing
   15 /* Ox           */   PULSE      50       30      "Ox"
   12 /* Wsh A        */   PULSE      50        0      "Flush system with Wsh A"
$Capping
   13 /* Caps         */   PULSE      25        0      "Caps to column"
   12 /* Wsh A        */   PULSE      50        0      "Wsh A"
   12 /* Wsh A        */   PULSE     150        0      "End of cycle wash"
```

MP($R_p$)/DE -- 5 μM

| Function | Mode | Amount /Arg1 | Time(sec) /Arg2 | Description |
|---|---|---|---|---|
| SDeblocking | | | | |
| 144 /* Advance Frac */ | NA | 1 | 0 | "Event out ON" |
| 0 /* Default */ | WAIT | 0 | 1.5 | "Wait" |
| 141 /* Photometer S */ | NA | 1 | 1 | "START data collection" |
| 16 /* Dblk */ | PULSE | 10 | 0 | "Dblk to column" |
| 16 /* Dblk */ | PULSE | 200 | 49 | "Deblock" |
| 38 /* Wsh A to Cl */ | PULSE | 80 | 0 | "Flush system with Wsh A" |
| 141 /* Photometer S */ | NA | 0 | 1 | "STOP data collection" |
| 39 /* Gas A to Cl */ | PULSE | 10 | 0 | "Gas A to Cl waste" |
| 144 /* Advance Frac */ | NA | 2 | 0 | "Event out OFF" |
| 12 /* Wsh A */ | PULSE | 200 | 0 | "Wsh A" |
| SCoupling | | | | |
| 1 /* Wsh */ | PULSE | 10 | 0 | "Flush system with Wsh" |
| 2 /* Act */ | PULSE | 10 | 0 | "Flush system with Act" |
| 18 /* A + Act */ | PULSE | 5 | 0 | "Monomer + Act to column" |
| 18 /* A + Act */ | PULSE | 18 | 60 | "Couple monomer" |
| 2 /* Act */ | PULSE | 3 | 10 | "Couple monomer" |
| 1 /* Wsh */ | PULSE | 7 | 56.1 | "Couple monomer" |
| 1 /* Wsh */ | PULSE | 50 | 0 | "Flush system with Wsh" |
| SOxidizing | | | | |
| 17 /* Aux */ | PULSE | 50 | 30 | "Aux" |
| 12 /* Wsh A */ | PULSE | 50 | 0 | "Flush system with Wsh A" |
| SCapping | | | | |
| 13 /* Caps */ | PULSE | 25 | 0 | "Caps to column" |
| 12 /* Wsh A */ | PULSE | 50 | 0 | "Wsh A" |
| 12 /* Wsh A */ | PULSE | 150 | 0 | "End of cycle wash" |

Applying one or more of these coupling routines with the appropriate dimer or monomer synthons, one skilled in the art can recognize that each of the chimeric oligomers described in subsequent examples can be synthesized.

Deprotection and purification of each chimeric oligomer was done essentially as described in Examples 8 through 12.

The identities of certain chimeric oligomers made according to this Example, as well as other compounds, were confirmed by electrospray mass spectrometry as shown in the following table:

| Seq. # | Sequence | Backbone | MW Predicted | MW Found |
|---|---|---|---|---|
| 2624-1 | 3'-CTGTTG TACGT ACCTTCTG-5' | Racemic MP | 5725 | 5726 |
| 2571-1 | 3'-CTGTTG TACGT ACCTTCTG-5' | 75%MP($R_p$) | 5725 | 5725 |
| 3130-3 | 3'-CCTGTTG TACGT ACCTTCTG-5' | MP($R_p$)/DE | 6028 | 6029 |
| 2566-1 | 3'-CCTGTTG TACGT ACCTTCTG-5' | PS | 6354 | 6357.9 |
| 2567-1 | 3'-CCTGTTG(TACGT)ACCTTCTG-5' | [MP][DE][MP] | 6022 | 6018 |
| 2687-1 | 3'-CCTGTTG(TACGT)ACCTTCTG-5' | [75%$R_p$MP][DE][75%$R_p$MP] | 6022 | 6022 |
| 3169-1 | 3'-CCTGTTG(TACGT)ACCTTCTG-5' | [MP($R_p$)/DE][DE][MP($R_p$)/DE] | 6033 | 6034 |
| 3214-1 | 3'-CCTGTTG(TACGT)ACCTTCTG-5' | [MP($R_p$)/DE][PS/DE][MP($R_p$)/DE] | 6082 | 6081 |
| 3257-1 | 3'-CCTGTTG(TACGTAC)CTTCTG-5' | [MP($R_p$)/DE][PS/DE][MP($R_p$)/DE] | 6100 | 6100 |
| 3256-1 | 3'-CCTGTTG(TACGT)ACCTTCTG-5' | [MP($R_p$)/DE][PS][MP($R_p$)/DE] | 6113 | 6114 |
| 3258-1 | 3'-CGTCCTCGATT(CCTTC)GATGGTAC-5' | [MP($R_p$)/DE][PS/DE][MP($R_p$)/DE] | 7300 | 7299 |
| 3260-1 | 3'-CGTCCTCGATT(CCTTC)GATGG TAC-5' | [MP($R_p$)/DE][PS][MP($R_p$)/DE] | 7331 | 7331 |
| 3261-1 | 3'-CTCTTCTTCTA(GTGAC)CTATATGG-5' | [MP($R_p$)/DE][PS/DE][MP($R_p$)/DE] | 7313 | 7310 |
| 3262-1 | 3'-CTCTTCTTCTA(GTGAC)CTATATGG-5' | [MP($R_p$)/DE][PS][MP($R_p$)/DE] | 7345 | 7346 |
| 3269-1 | 3'-ACGTCTGATCA(GTAAC)TAACTCAC-5' | [MP($R_p$)/DE][PS/DE][MP($R_p$/DE]) | 7309 | 7308 |
| 3270-1 | 3'-ACGTCTGATCA(GTAAC)TAACTCAC-5' | [MP($R_p$)/DE][PS][MP($R_p$)/DE] | 7341 | 7340 |

1. (Parenthesis) refers to the portion that activates RNAseH; the linkage on the 3'-side of the indicated nucleoside is charged.

Example A

Preparation of Plasmid Expressing a Polycistronic E6/E7 mRNA

An expression vector having an insert coding for HPV11 E6/E7 was prepared using the expression vector pRc/CMV (Invitrogen) as follows:

The plasmid pRC/CMV was linearized with *Hind* III. The recessed 3' ends were filled with the 5'-3' polymerase activity of $T_4$ DNA polymerase.

A full length clone of HPV-11 cloned at the *BamH I* Site in pBR322 was digested with the restriction enzymes *Bst* EII and *Hinf* I. The 873 base pair fragment containing the E6 and E7 open reading frames was purified on agarose gel. The restriction ends of this fragment were modified by filling in the recessed 3'-ends with $T_4$ DNA polymerase.

The vector and insert were ligated with $T_4$ DNA ligase and transformed into DH5α *E. Coli*. recombinants were screened for appropriate insert and orientation as well as E6/E7 transcription and translation activity.

This plasmid (pRc/CMV11-E6/E7) was used to prepare the polycistronic mRNA used in the cell free translation system described in Example F.

Example B

Preparation of Plasmid Having Monocistronic E7 Insert

An expression vector having an HPV-11 E7 insert was prepared using pcDNA-1 (Invitrogen) as follows.

The plasmid pcDNA was digested with *Bam*H I and with *Xba* I. A fragment containing the complete open reading frame of HPV-11 (from -30 till the termination codon) flanked by *Bam* HI and *Xba* I restriction sites was prepared by PCR using standard protocols. The digested vector and fragment were ligated with T4 DNA ligase and transformed into MC 1061/P3 cells. Recombinants were screened for appropriate insert, transcription and translation.

This plasmid (pcDNA E7) was used to prepare the monocistronic mRNA used in the cell-free translation system described in Example G and in the transient expression assay in COS-7 cells described in Example J and in the RNase H cleavage assay of Example I.

Example C

Preparation of Plasmid Having a E1 Insert

An expression vector having an HPV-11 E1 insert was prepared using the expression vector pRc/CMV (Invitrogen) as follows.

The plasmid pRC/CMV was linearized with *Hind* III. The recessed 3' ends were filled in with $T_4$ DNA polymerase, and then the plasmid was cut with *Xba* I.

A full length clone of HPV-11 cloned at the *Bam* HI site in pBR322 was digested with the restriction enzyme *Apa*L I. The recessed 3'-ends were filled in with the 5'-3' polymerase activity of the Klenow fragment of DNA polymerase I. The modified DNA was next cut with *Spe* I and a 2428 base pair fragment containing the complete E1 ORF was agarose gel purified.

The modified vector and E1 insert were ligated with $T_4$ DNA ligase and transformed into DH5$\alpha$ *E. Coli*.

Recombinants were screened for appropriate insert, transcription and translation.

This plasmid (pRc/CMVII-E1) was used in the cell free translation system of Example M.

Example D

Preparation of Plasmid Having an E2 Insert

An expression vector having an HPV-11 E2 insert was prepared using pRc/CMV (Invitrogen) as follows.

The plasmid pRC/CMV was linearized with *Hind* III, followed by treatment with calf thymus alkaline phosphatase.

To isolate the E2 open reading frame, a full length clone of HPV-11, cloned at the *Bam* HI site in pBR322 was digested with the restriction enzymes *Xmm I* and *Ssp I*. The recessed 3' ends were filled in with the 5'-3' polymerase activity of the Klenow fragment of DNA polymerase I. *Hind III* linkers were then added. The 1309 base pair fragment containing the complete E2 ORF was agarose gel purified.

The modified vector and E2 insert were ligated with $T_4$ DNA ligase and transformed into DH5$\alpha$ *E. Coli*. Recombinants were screened for appropriate insert, transcription and translation.

This plasmid (pRc/CMVII-E2) was used in the cell-free translation system of Example O.

Example E

Evidence for Pseudoknot Structure Upstream of Translation Initiation Codon of HPV E7

Phylogenetic analysis was used to determine the secondary structure of HPV E7 mRNA from N380 to N580 (HPV-11 sequence numbers). Sequence fragments of 13 different HPV types were aligned using a computer algorithm. The alignments were further adjusted by hand to give the final alignment.

The secondary structures generated with this analysis were consistent with only one structure: a pseudoknot just upstream of the translation initiation codon of E7. The pseudoknot found here is a hairpin stem-loop which forms an additional helix using bases in the loop. This structure is best defined by HPV-44, -43, -11, and -6b, all them associated with genital warts. Similar structures with lower levels of stability were also found in the other HPV types analyzed.

Figures 2A and 2B explain the pseudoknot structures for HPV-6b and HPV-11. Please note that the AUG in boldface is the start codon for the E7 mRNA. The pseudoknot starts 6 nucleotides upstream from the from the HPV-11 E7 AUG. In the case of high risk HPVs a variable number of nucleotides is present between the pseudoknot structure and the initiation codon of E7.

It is interesting to note that in low risk HPVs as well as in HPV 2a, the E6 and E7 proteins are encoded by a polycistronic mRNA, and that the reading frames of both proteins overlap each other by about 20 bases (*see* Figure 1A). Since the reading frame for E7 is -1 (or +2) as compared to E6, this pseudoknot may be involved in the ribosomal frame shifting needed for translation of both proteins, or perhaps in regulation of the level of E6/E7 protein production. Pseudoknots have been shown to have both of these functionalities in other systems. Interestingly, in the case of high risk HPVs where E6 and E7 do not overlap, and in which E7 is generated from a spliced E6/E7 mRNA, a variable number or nucleotides is present between the structure and the initiation codon of E7.

Example F

Demonstration of Activity of Antisense Oligomers Targeted to HPV-11 E7 in Cell Free Translation Extracts

Mono-cistronic (100 nM) HPV-11 E7 or polycistronic (50 nM) HPV-11 E6/E7 RNA was co-translated with chloramphenicol acetyl transferase (CAT) RNA (2 to 10 nM) in cell-free rabbit reticulocyte extracts (Promega). The contents of each assay system was as follows.

| COMPONENT | FINAL CONCENTRATION |
|---|---|
| *In vitro* transcribed uncapped RNA | (As noted above) |
| $^{35}$S-cysteine | 0.8 mCi/mL |
| Amino acids mixture, cysteine deficient | 20$\mu$M each |
| Rabbit reticulocyte lysate | 72% by volume |
| RNAsin (Promega) | 0.5 units /$\mu$L |
| Oligomer | 1 to 10$\mu$M |

Cell free translation was performed at 37°C for 60 minutes and was stopped by addition of SDS gel loading buffer and incubation at 95° for 3 minutes.

Translation of E7 was evaluated after immuno-precipitation with $\alpha$E7 goat anti-serum and protein A sepharose, followed by SDS-PAGE and phospho-image analysis. This protocol was used in the cell-free translations of Examples G and L.

Selection of target sequence for low risk HPV E7

The effect of phosphodiester oligonucleotides 2406-1 [SEQ. ID. NO. 6], 2552-1 [SEQ. ID. NO. 7], 2458-1 [SEQ. ID. NO. 8], 2457-1 [SEQ. ID. NO. 9], 2549-1 [SEQ. ID. NO. 10], 2553-1 [SEQ. ID. NO. 11], 2518-1 [SEQ. ID. NO. 12], 2525-1, [SEQ. ID. NO. 13], 2498-1 [SEQ. ID. NO. 14], 2492-1 [SEQ. ID NO. 15], 2589-1 [SEQ. ID. NO. 16] and 2590-1 [SEQ. ID. NO. 17] (*see* Table I) on cell-free translation of polycistronic HPV-11 E6/E7 mRNA was evaluated using the protocol described above. Translations were made in the presence 0.02 units/$\mu$l of RNAseH, and oligonucleotide at either 0.01, 0.1 or 1 $\mu$M. CAT mRNA was co-translated as negative control. Please note that all the oligonucleotides tested, but 2589-1

and 2590-1, and 2492-1 are fully complementary to HPV-11 as well as the HPV-6b sequence.

Table I summarizes the results we obtained. The percentages of inhibition were calculated as the difference between the inhibition of E7 protein synthesis and control CAT translation. For example, in cases where no specific inhibition was indicated, no differences between inhibition of E7 and CAT was observed. Overall, the results presented here showed that the specific inhibition of the oligonucleotides increased as the target sequence was located downstream from N542 (AUG-7), indicating that HPV-11 mRNA sequences present downstream from N542 (AUG-7) are accessible to oligonucleotides.

Because oligonucleotide 2498-1 complements HPV-6b as well as HPV-11 and showed good specific translation inhibition of E7, it was selected as our preferable oligomer sequence.

Finally, the results presented here add support to the presence of a pseudoknot just upstream from the translation initiation site of E7 (as described in Example E), since the phosphodiester data shows a decrease in oligomer activity as the oligomer overlaps with the pseudoknot.

Results are reported in Tables I and VI.

Example G

Inhibition of Cell-Free Translation of Moncistronic E7 or Polycistronic E6/E7 mRNA with RNaseH Mediated Cleavers or With Steric Blockers.

Since HPV-11 E7 may be translated from a polycistronic E6/E7 mRNA (Figure 1A) or a monocistronic E7 mRNA (Figure 1B), it was important to compare the antisense activity of oligomers on both mRNAs. In particular, it was of interest to know the ability to inhibit translation of the polystronic mRNA, as compared

to the monocistronic mRNA, by RNase H-mediated cleavers or steric blockers. Oligonucleotide 2567-1 [SEQ. ID. NO. 26], a chimeric methylphosphate oligomer was used as an example of a RNase H-mediated cleaver, and oligonucleotide 2644-1 [SEQ. ID. NO. 18], a 2'-OMeRNA, was used as an example of a steric blocker. HPV-11 E7 monocistronic mRNA (circles) or E6/E7 polycistronic mRNA (squares) were translated in rabbit reticulocyte lysates in the absence or in the presence of different concentrations of [MP][DE]$_5$[MP] oligonucleotide 2567-1, or 2'OMeRNA oligonucleotide 2644-1, as described in Example F. RNase H at 0.04 units/ul was present in the translations run in the presence of oligonucleotide 2567-1, and CAT mRNA, at 10 nM, was co-translated as a negative control (triangles) in both cases.

Analysis of the data presented in Figure 3A indicated that oligonucleotide 2567-1 was able to inhibit E7 translation whether E7 was translated from the monocistronic (circles) or from the polycistronic mRNA (squares). The oligomer was only slightly more active on the monocistronic mRNA than on the polycistronic mRNA. Figure 3B depicts the results obtained with oligonucleotide 2644-1. This result showed that the activity of the steric blocker on either mRNA was lower than the activity observed with the RNase H mediated cleaver 2567-1, and that oligomer 2644-1 produced very little inhibition of E7 synthesis when it is translated from the polycistronic mRNA. In the case of both the oligomers, no effects on CAT translation were observed, indicating that their was activity was very specific.

Overall these results indicated that inhibition of HPV E7 translation will be more successfully achieved by the use of RNase H medicated cleavers.

Example H

Determination of Hybridization of Antisense Oligomers to RNA

The melting temperatures (Tm) for RNA duplex forming antisense oligomers were determined at equimolar concentrations (1.2 μM) of oligomer and synthetic RNA target in 1 ml of buffer containing 20 mM $DPO_4$, pH 7.2, 0.1 mM NaCl, 0.1 mM EDTA and 0.03% sarkosyl. The reaction mixtures were heated by 80°C and then slowly cooled to 4°C over approximately 4 to 6 hours. The annealed samples were then transferred to 1 cm quartz cuvettes; absorbance at 260 nm as a function of temperature was monitored using a Varian Cary Model 3E Spectrophotometer containing a 6 X 6 temperature-controlled sample holder interfaced with an IBM compatible PC computer. The temperature was varied from 5°C to 80°C at a ramp rate of 1°C/minute. The Tm for each melt profile was defined at the point corresponding to the first derivative (of the $A_{260}$-temperature function).

Results are reported in Tables VI and IX.

Example I

Demonstration of Activity of Antisense Oligomers in a Cell-Free RNAse H Cleavage Assay

*In vitro* transcribed, uncapped mono-cistronic RNA was prepared by transcribing plasmid pcDNA11E7 with RNA polymerase (Ambion MegaScript).

The E7 RNA was incubated at a concentration of 100 nM in the presence of 0.04 units μL *E. Coli*. RNAse H (Promega), 3.5 mM $MgCl_2$, 25 mM KCl, 70 mM NaCl and 20 mM potassium acetate at 37°C for 30 minutes. Reactions were stopped by addition of formamide gel loading buffer followed by heating to 100°C for 5 minutes.

Samples were analyzed by 4% Urea-PAGE analysis, followed by staining with ethidium bromide.

Percentages of cleavage of E7 MRNA, in the presence of RNAse H, of methylphosphonate chimeric oligomers 2567-1 [SEQ. ID. NO. 26], 3169-1 [SEQ. ID. NO. 29], 3214-1 [SEQ. ID. NO. 30], 3257-1 [SEQ. ID. NO. 31], 3341-1 [SEQ. ID. NO. 33], and 3336-1 [SEQ. ID. NO. 34], are shown in Table XIV. Dose response effects were obtained for all the oligomers at the concentrations tested. The order of potency was 3169-1 ~= 3257-1 > 3214-1 ~= 2567-1 > 3336-1 > 3341-1. All oligomers showed good specificity, cleaving E7 mRNA in one position.

Results are reported in Table XIV.

Example J

Demonstration of Activity of Antisense Oligomers in Transiently Transfected COS-7 Cells

COS-7 cells (obtained from ATCC [Catalog CRL 1651]) were seeded at 1 X $10^5$ cells/well in 24 well plates and then cultured overnight in cell culture media (90% DMEM, 10% fetal bovine serum and 50 I.U./ml penicillin, 50 mg/ml streptomycin and 0.25 μg/ml amphotericin B). After 24 hours the cells were approximately 80 to 90% confluent.

A transfection cocktail of 2.5 μg/mL pcDNA1E7, 50 μg/mL transfectam (Promega) and varying concentrations of oligomer was prepared and incubated for 15 minutes at room temperature after a 2 second vortex mix.

Cells were washed on the plates two times, 1 ml/well with Optimem (Gibco-BRL). Then 0.5 mL transfection cocktail per well was applied to duplicate wells. The plates were incubated for 4 hours in 5% $CO_2$ at 37°C. After incubation cells were washed two times, 1 mL/well with cell culture media and cultured overnight. Then cells were washed twice, 1 mL/well with cysteine deficient DMEM and then incubated for 30 minutes in cysteine deficient DMEM under cell culture conditions. Cells were labelled by incubation with 250 μCi of $^{35}S$-

cysteine/well in 500 μL cysteine deficient DMEM without serum for 5 hours. The cells were then washed twice, 1 mL/well with 1X phosphate buffered saline and then lysed with 100 μL SDS sample buffer (50 mM Tris-Cl [pH 6.8], 100 nM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol). Wells were washed with 100 μL RIPA buffer (10 mM Tris-Cl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate) and combined with sample buffer lysate.

E7 synthesis was evaluated by immunoprecipitation of E7 protein with goat anti-HPV-11 E7 serum and protein A sepharose beads (Sigma). Immunoprecipitated E7 protein was quantitated by SDS-PAGE and phospho-image analysis. Total protein synthesis was evaluated by SDS-PAGE and phospho-image analysis of a fraction of the transfected cell lysate before immunoprecipitation.

Total protein synthesis was analyzed by SDS-PAGE separation of an aliquot of the cell extract, autoradiography and phospho-image quantitation of all the proteins present in each lane. Results are expressed as percentage of protein translation respect to the value of translation obtained in the absence of oligonucleotide.

Results of a representative experiment performed with the methylphosphonate chimeric oligomer 3256-1 [SEQ. ID. NO. 32] is shown in Figure 5. In addition, Table VII summarizes the IC50 and IC90 values obtained with chimeric oligomers 3169-1 [SEQ. ID. NO. 29], 3214-1 [SEQ. ID. NO. 30], 3257-1 [SEQ. ID. NO. 31], 3256-1 [SEQ. ID. NO. 32], 3327-1 [SEQ. ID. NO. 77] and 3336-1 [SEQ. ID. NO. 34].

It is clear from this example that chimeric oligonucleotides 3214-1, 3257-1 and 3256-1, which contain all phosphorothioate ([PS]) or alternating phosphorothioate/phosphodiester ([PS/DE]) linkages in

the middle and chiral methylphosphonate dimers linked by phosphodiester linkages ([Rp-MP/DE]) as end-blocks are potent inhibitors of transient expression of HPV E7 protein in COS-7 cells.

Chimeric oligonucleotides with phosphodiester linkages in the middle, such as 3169-1, were not potent in the cell-based assay, although they proved to be very potent in the cell-free assay (Table VI). This difference may be due to the intracellular instability of the phosphodiester linkage.

Finally, oligonucleotides containing 2'OMe modification in the sugar of the nucleosides present at the ends (oligonucleotides 3327-1 and 3336-1) were less potent than the corresponding chimeras with [Rp-MP/DE] ends.

Results are reported in Table VII and Figure 5.

Example K

Chimeric Methylphosphonate Oligomers Produced Reduction of HPV-11 E7 mRNA Levels in COS-7 Cells

To prove that the downregulation of E7 protein levels produced by the chimeric oligomers in cells was the result of an antisense interaction with its mRNA (due to a RNase H mediated cleavage of the oligomer-RNA duplex), the intracellular levels of E7 mRNA after treatment of transient transfected cells with a chimeric oligomer were determined using a RNase protection assay. The RNA probe used in the assay complements HPV-11 N564 to N841, so that if E7 mRNA is present, a protected band of 278 nucleotides should be expected. As control for the RNase protection assay, we determined that the Protection was specific for E7 mRNA, as no protection to the probe was rendered by incubation of it with 10 ug of yeast RNA or by incubation with RNA from mock-transfected cells. Moreover, the signal was present after DNase treatment of the samples prior to the RNase

protection assay, denoting protection of the probe due to E7 mRNA rather than to E7 cDNA.

The phosphoimage presented in Figure 6A, shows that treatment of the cells with oligomer 3256 [SEQ. ID. NO. 32] at 0.05, 0.5 or 5 uM produced a dose-dependent decrease in the amount of E7 probe (lanes 1-4), indicating a reduction in the amount of E7 mRNA present in the cell extracts. Treatment of the cells with 5 uM of an oligomer targeted to HPV-11 E2 [SEQ. ID. NO. 76] did not significantly reduce the amount of E7 mRNA present (lane 5), denoting that the reduction in E7 mRNA was sequence-specific. Moreover, probing of the same oligomer-treated cell extracts with a probe targeted to GAPDH mRNA showed no reduction in the probe after the RNase treatment, indicating no effect of oligomer 3256 on this cellular mRNA (Figure 6B). Overall these results confirm that the E7 mRNA reduction produced by oligomer 3256 was specific and demonstrate that the reduction in E7 protein levels is due to a specific interaction of the oligomer with the E7 mRNA.

Example L

Inhibition of HPV-11 E6 as well as HPV-11 E7 Cell-free Synthesis with One Single RNaseH Medicated Cleaver Targeted to the Translation Initiation Codon of E7

As shown in Figure 1A, HPV-11 E6 proteins are translated from the polycistronic E6/E7 mRNA containing the ORF of E6 upstream from the ORF of E7. Since oligomers targeted to the translation initiation codon of E7 will also complement the 3' portion of the ORF of E6, it was important to determine the activity of these oligomers on E6 production.

HPV-11 E6/E7 polycistronic mRNA was translated in rabbit reticulocyte lysates in the absence or in the presence of different concentrations of oligomers. Translation reactions were carried out at 37°C, in the

presence of RNAse H at 0.04 units/ul. CAT mRNA, at 10 nM, was co-translated as negative control in both cases. The effect of the oligomers on E7 translation (circles) was evaluated after immuno-precipitation of the translation reaction with αE7 antiserum, size fractionation on SDS PAGE and phospho-image analysis. The effect of the oligomers on E6 translation (squares) or CAT translation (triangles) was evaluated after size fractionation by SDS PAGE of one aliquot of the reaction mixture followed by phospho-image analysis. Results are expressed as percent of protein translation with respect to the value of translation obtained for each protein in the absence of oligonucleotides.

Analysis of the activity of steric blockers or RNase H mediated cleavers indicated that while steric blockers had no significant effect on E6 production, RNase H mediated cleavers significantly inhibited E6 production in the cell-free assay. As an example, Figures 4A and 4B depict the results obtained with phosphodiester oligonucleotide 2498-1, [SEQ. ID. NO. 14] (Figure 4A) and with [MP][DE]$_5$[MP] oligomer 2567-1, [SEQ. ID. NO. 26] (Figure 4B).

Example M

In vitro Inhibition of HPV-11 E6 Protein Expression With Chimeric Methylphosphonate Oligomers Targeted to the E7 Translation Start Site

HPV-11 E6 is translated from a E6/E7 mRNA containing the E6 ORF upstream from the E7 ORF (Figure 1). Consequently, oligonucleotides that are targeted to the translation initiation codon of E7 (HPV-11 bases 532-542) will also complement the 3' portion of the E6 ORF. Since our previous results indicated that the inhibition of E7 production in cells is due to an interaction between the oligomer and the mRNA (Example K) and that oligomers targeted to E7 diminished the amount of E6

synthesized in a cell-free reaction (Example L), it was important to determine the activity of oligonucleotides targeted to the E7 translation start on E6 production in cells.

To examine whether chimeric oligonucleotides targeted to E7 could also block expression of E6, plasmids pcDNA11E7, or pED/E6 [plasmid pED/E6 contains the HPV-11 E6 gene plus 32 upstream bases (HPV nucleotides 72-555) behind the adenovirus major late promoter and tripartite leader], encoding either E7 or E6, were transfected into COS-7 cells together with chimeric oligomer 3256 [SEQ. ID. NO. 32] at 5 uM.

Levels of E6 and E7 protein were evaluated after 18 hours by metabolic labeling and precipitation with specific anti-E7 or anti-E6 serum, followed by autoradiography. As shown in Figure 7, oligomer 3256 inhibited expression of E7 (lane f) as expected. This oligonucleotide also blocked expression of E6 (lane c). A control chimeric oligomer 3218 [SEQ. ID. NO. 46] targeted to the translation start site of the E1 protein of HPV-11 had no effect on expression of E7 (lane g) and little effect on E6 (lane d). These results agree with the cell-free data and indicate that oligomer 3256 in addition to the previous observed inhibition of E7, was able to produced downregulation of E6 protein production.

It is clear that chimeric oligonucleotides are effective when targeted to either the translation start site of an mRNA (E7 in this case) or to the 3' end of an ORF (E6). This results supports the idea that chimeric oligonucleotides can block synthesis of multiple proteins encoded in polycistronic mRNAs.

Example N

Demonstration of Oligomer Activity by Microinjection in VERO Cells

(i) Micro injection

Oligomers were microinjected together with E2 (pRc/CMV 11-E2) or E7 (pcDNA E7) expression plasmids at 50 $\mu g/\mu l$ into the cytoplasm of VERO cells according to the following procedure. On the day preceding injection, VERO cells (approximately 2 X $10^5$ cells/ml) were plated on coverslips. Plasmid DNA was diluted in PBS to a concentration of 20 $ng/\mu l$ (E7) or 50 $ng/\mu l$ (E2) in an eppendorf tube. The tubes containing plasmid DNA were centrifuged for 15 minutes at 1,400 rpm. the tubes were set on ice prior to microinjection. A 2 $\mu L$ aliquot of plasmid DNA solution was loaded onto a femto top. The tip was set with coverslip at 45°C, the pressure on the microinjector was set at 80 and the injection was performed. The coverslips were incubated at 37°C overnight after injection. At 16 hours post-injection, cells were fixed and immunostained with goat anti-E7 polyclonal antibody, as explained below.

(ii) Indirect Fluorescence Immunoassay

Expression level of E2 or E7 was assessed using a fluorescent antibody assay as follows.

Coverslips were fixed in 10% formaldehyde in PBS for 20 minutes at room temperature and then washed twice with PBS.

Prior to use in this assay, goat anti-HPV-11 E7 or HPV-11 E2 serum was preabsorbed with VERO cells as follows. Confluent VERO cells from two T-150 flasks were scraped and then washed twice with PBS. then 200 $\mu l$ serum was added to the cell pellet and mixed at 40°C overnight. the mixture was centrifuged; the supernate was removed to a new tube. the preabsorbed serum was stored in 50% glycerol at -20°C.

Coverslips were incubated with goat anti-HPV-11 E7 or HPV-11 E2 protein serum preabsorbed as set forth below at a 1:1000 dilution in PBS for two hours at room temperature. Coverslips were washed with PBS three times, five minutes per wash. Coverslips were incubated with FITC-conjugated Donkey Anti-Goat IgGAb (Jackson, ImmunoResearch, Cat #705-095-147) at 1:200 dilution in PBS. Coverslips were washed with PBS three times and then air-dried. Coverslips were mounted with 50% glycerol on slide glass. Coverslips were then examined under UV lights.

Results are reported in Tables VI, VII, and XII.

Example O

Demonstration of Activity of Antisense Oligomers Targeted to E2 in Cell-Free Translation Extracts

E2 RNA was prepared by transcribing plasmid pRc/CMV-11E2 with T7 RNA polymerase using an Ambion MegaScript kit, following the manufacturer's directions.

*In vitro* transcribed E2 mRNA was cell-free translated in rabbit reticulocyte lysates (Promega). The final concentrations of each component of the assay system was as follows:

| | |
|---|---|
| In vitro transcribed uncapped RNA: | 50 nM |
| $^{35}$S-Methionine: | 1.3 uCi/ul |
| Potassium Acetate: | 20 mM |
| Amino acid mixtures, methionine deficient: | 50 uM |
| Rabbit Reticulocyte Lysate: | 33 % vol/vol |
| RNAsin: | None or 0.5 units/ul |

Cell-free translation was performed at 37°C for one hour and was stopped by addition of SDS gel loading buffer and incubation at 95°C for 3 minutes. Translation of E2 was evaluated after separation of the translation mix by SDS-PAGE, followed by phospho-image

analysis. To determine the effect of oligomers targeted to the translation initiation codon of E2, *in vitro* transcribed E2 mRNA was translated in the presence of 0.02 or 0.04 units/ul of RNAse H, and using oligonucleotide concentrations ranging from 0.01 to 10 uM. CAT mRNA was co-translated, or translated in independent translation reactions as control.

As shown in Tables X and XI, different levels of inhibition of E2 cell-free translation and of specificity regarding the CAT control mRNA were obtained with the oligomers tested. Overall, the results indicated that in most cases, steric blockers were significantly less potent than RNase H activators. (Compare the activity of oligomer 3102-1 [SEQ. ID. NO. 52] with 3129-1 [SEQ. ID. NO. 64] or 3124-1 [SEQ. ID. NO. 57]; or the activity of 3103-1 [SEQ. ID. NO. 53] with 3173-1 [SEQ. ID. NO. 55]. In addition, end-blocked chimeric oligomers were more specific than all phosphodiester oligomers (compare the activity and specificity of oligomer 3102-1 [SEQ. ID. NO. 52] with 3233-1 [SEQ. ID. NO. 60] or 3234-1 [SEQ. ID. NO. 61]).

Our results using the cell-free translation assay indicated that the region of the E2 mRNA around the translation initiation codon, from AUG-12 to AUG+16, was accessible to antisense targeting, and that oligonucleotide backbone influenced the degree of antisense activity and specificity of the oligomer.

Example P

Demonstration of Activity of Antisense Oligomers Targeted to E1 in Cell-Free Translation Extracts

E1 mRNA was prepared by transcribing plasmid pRc/CMV-11E1 with T7 RNA polymerase using an Ambion MegaScript kit or an Ambion Message Machine kit, following the manufacturer's directions.

In vitro transcribed E1 mRNA was cell-free translated in wheat germ extracts (Promega). The final concentrations of each component of the assay system was as follows:

| | |
|---|---|
| In vitro transcribed E1 mRNA: | 100 nM (uncapped) or 15 nM (capped) |
| $^{35}$S-Methionine: | 1 uCi/ul |
| Potassium Acetate: | 75 mM |
| Amino acid mixtures, methionine deficient: | 80 uM each |
| Wheat Germ Extract: | 50 % vol/vol |
| RNAsin: | 0.5 units/ul |
| Oligomers: | 0.01 to 10 uM |

Cell-free translation was performed at room temperature for one hour and was stopped by addition of SDS gel loading buffer and incubation at 95°C for 3 minutes. Translation of E1 was evaluated after separation of the translation mix by SDS-PAGE, followed by phospho-image analysis.

To determine the effect of oligonucleotides targeted to the translation initiation codon of E1 and to the splice donor for E2, E5a, E5b and E1i^E4 present at N847, *in vitro* transcribed E1 mRNA was translated in the presence or absence of oligomers 2555-1 [SEQ. ID. NO. 40], 2556-1 [SEQ. ID. NO. 41], 2557-1 [SEQ. ID. NO. 42], 2744-1 [SEQ. ID. NO. 47], 3105-1 [SEQ. ID. NO. 43], 3106-1 [SEQ. ID. NO. 44], 3196-1 [SEQ. ID. NO. 45] and 3218-1 [SEQ. ID. NO. 46] (*see* Table III). Translations were performed in the presence of 0.02 units/ul of RNAse H, and using oligomer concentrations ranging from 0.01 to 10 uM. CAT mRNA was translated in an independent translation reaction as control.

As shown in Table IX, steric blocker oligomers 3105-1 and 3196-1 showed low potency, and high specificity,

while phosphodiester 2555-1, 2556-1, and 2557-1 produced high levels of inhibition of E1 translation at 1 uM, and different levels of non-specific translation inhibition of the CAT control. Among the phosphodiesters, oligomer 2557-1 (targeted to the splice donor site at N847) showed less non-specific effects and oligonucleotide 2744-1, a 2'-OMe end-capped diester, was the most potent and specific.

According to these results, the preferred target sequence around the translation initiation codon of E1 was AUG+14 and AUG+33 regarding oligomer backbones, a chimeric backbone is preferable.

Example Q

Demonstration of Activity of Antisense Oligomers Targeted to E6 in Cell-Free Translation Extracts

Polycistronic E6/E7 mRNA was prepared by transcribing the plasmid pRc/CMV11-E6/E7 with T7 RNA polymerase using an Ambion MegaScript kit, following the manufacturer's directions.

*In vitro* transcribed E6/E7 mRNA (50nM) was cell-free translated in rabbit reticulocyte lysates (Promega) as described in Example F. Cell-free translation was performed at 37°C for one hour and was stopped by addition of SDS gel loading buffer and incubation at 95°C for 3 minutes. Translation of E6 was evaluated after separation of the translation mix by SDS-PAGE analysis, followed by phospho-image analysis.

To determined the effect of oligomers targeted to the translation initiation codon of E6, *in vitro* transcribed E6/E7 mRNA was translated in the presence or absence of the oligonucleotides showed in Table V. Translations were performed in the presence of 0.02 or 0.04 units/ul of RNAse H, and using oligomer concentrations ranging from 0.01 to 10 uM. CAT mRNA was co-translated as control. As shown in Table XIII,

different levels of inhibition of E6 cell-free translation were obtained with the oligomers tested. In general, low levels of inhibition of E6 translation were obtained, which suggested that the region of the E6 translation initiation codon, from AUG-10 to AUG+21, presented poor accessibility to antisense targeting. The best results were obtained with oligomer 3215-1 [SEQ. ID. NO. 76], a 20-mer chimeric methylphosphonate oligomer targeted to AUG-10.

Example R

Downregulation of HPV-16 E7 mRNA levels in HPV-transformed CaSki cells treated with [Rp-MP/DE][PS][Rp-MP/DE] oligomer 3678 as compared with a Control [Rp-MP/DE][PS][Rp-MP/DE] oligomer

CaSki cells (obtained from ATCC, Catalog CRL 1550) were seeded at 4 X $10^5$ cells/60 mm dish and cultured in cell culture medium (90% RPMI, 10% fetal bovine serum, 50 I.U./mL penicillin and 50 mg/mL streptomycin). After 28 hours the cells were approximately 50% confluent.

A transfection mix containing 18 μg/mL of Lipofectamine (BRL) and various concentrations of oligomer was prepared, vortex mixed, and incubated for 15 minutes at room temperature. Cells were washed two times with 4 mL/dish of OptiMem (Gibco BRL), and 2 mL of transfection mix per dish was applied to duplicate samples. The cells were incubated for 16 hours in 5% $CO_2$ at 37°C. After the transfection the cells were washed two times with 4 mL/well of PBS and total cytoplasmic RNA was extracted using RNAzol™B (TEL-TEST, Inc.) following the manufacturer's directions.

The intracellular level of HPV-16 E7 mRNA after the oligomer treatment was determined using a RNase protection assay. The RNA probe used in the assay complements HPV-16 N566 to N855, so that if E7 mRNA is present, a protected band of 289 nucleotides should be

expected. As control for specifity of the oligomer treatment, the intracellular levels of actin mRNA were determined by probing an aliquot of the intracellular RNA with a commercially available probe targeted to actin (Ambion, pTRI-β-actin-125-Human).

Phosphoimage quantitation of the HPV-16 E7 protected RNA bands indicated that treatment of the cells with oligomer 3678 [SEQ. ID. NO. 99] produced a dose-dependent reduction in the amount of E7 mRNA present in the cell extracts (*see* Figure 10). Maximum inhibition obtained was about 60% when the oligomer was present at 1 to 3 μM. Treatment of the cells with 3 μM of control oligomer targeted to the CAT poly(A) site [SEQ. ID. NO. 102] [5'CCTCATTTT(ATTAG)GAAAGGACAG-3'] did not significantly reduce the amount of E7 mRNA present, denoting that the reduction in E7 mRNA was sequence-specific. Moreover, probing of the same oligomer-treated cell extracts with a probe for actin mRNA showed no reduction in the levels of actin in cell extracts treated with 0.3 or 1 μM of oligomer 3678 [SEQ. ID. NO. 99], indicating specific antisense effect at these oligomer concentrations. Treatment of the cells with 3 μM of oligomer 3678 [SEQ. ID. NO. 99] or with control oligomer [SEQ. ID. NO. 102] showed about 40% inhibition of actin, indicating non-specific effects at these high concentrations. Overall these results showed that oligomer 3678 [SEQ. ID. NO. 99] specifically inhibits HPV-16 E7 mRNA levels in transformed CaSki cells.

Example S

Downregulation of HPV-16 E7 mRNA levels in HPV-transformed CaSki cells treated for three days with [Rp-MP/DE][PS][Rp-MP/DE] oligomers 3678, 3679 AND 3680

CaSki cells seeded in 60 mm dishes and cultured as described in Example R were transfected using 18 ug/mL of Lipofectamine (BRL) and 1 uM of oligomers 3678 [SEQ.

ID. NO. 99], 3679 [SEQ. ID. NO. 100], 3680 [SEQ. ID. NO. 101] or control oligomer [SEQ. ID. NO. 102], as described before. After this first transfection the cells were washed two times with 4 mL/well of cell culture medium, and incubated for 8 hours under tissue culture conditions. The cells were transfected for 16 hours and recover under tissue culture conditions two more times. After the third transfection the cells were washed two times with 4 mL/well of PBS and total cytoplasmic RNA was extracted as described before.

The intracellular levels of HPV-16 E7 mRNA after treatment with oligomers were determined using a RNase protection assay as described in Example R. As control for specificity, the intracellular levels of GAPDH mRNA were determined by probing an aliquot of the intracellular RNA with a commercially available probe targeted to GAPDH (Ambion, pTRI-GAPDH- Human).

Phosphoimage analysis of the HPV-16 E7 protected RNA bands indicated that treatment of the cells with oligomers 3678 [SEQ. ID. NO. 99], 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101] produced reduction in the amount of E7 mRNA present in the cell extracts (Figure 11). Inhibition obtained was about 70% for oligomer 3678 [SEQ. ID. NO. 99] and about 55% for oligomers 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101]. No significant reduction of GAPDH levels were observed, indicating specific inhibition. Treatment of the cells with control oligomer 3268 [SEQ. ID. NO. 102] [targeted to the CAT poly(A) 3268 site] produced approximately 40% inhibition of GAPDH and approximately 25% inhibition of HPV-16 E7, indicating some non-specific effects. Overall, these results indicated that oligomers 3678 [SEQ. ID. NO. 99], 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101] inhibit HPV-16 E7 mRNA levels in transformed CaSki cells. Oligomer 3678 [SEQ. ID. NO. 99] was the most potent in this model.

TABLE I

CORRELATION OF PHOSPHODIESTER OLIGO ACTIVITY WITH THE PROPOSED SECONDARY STRUCTURE OF HPV-11 E7 mRNA

a. Prediction of the secondary structure of HPV-11 E7 around the translation initiation codon (N469-N560)

```
          C
5'-AGGC-ACG UUC

   UUGC UGC GGGAAGGUGACC
      U   U
                  CUGCUGGACAACAUGCAUGGAAGACuUGUUACCCUAAAGGAUA-3'
```

b. Activity of phosphodiester antisense oligos:

| SEQ. ID NO. | SEQUENCE | INHIBITION |
|---|---|---|
| 6 [2406-1] | 3'-ATGTGACGACCTGTTGTA-5' | No spec. inh. |
| 7 [2552-1] | 3'-GTGACGACCTGTTGTACG-5' | No spec. inh. |
| 8 [2458-1] | 3'-ACCTGTTGTAC&GTA-5' | No spec. inh. |
| 9 [2457-1] | 3'-GACCTGTTGTACGTAC-5' | No spec. inh. |
| 10 [2549-1] | 3'-CGACCTGTTGTACGTACC-5' | ~20% spec. inh. |
| 11 [2553-1] | 3'-CCTGTTCTACGTACCTTC-5' | ~55% spec. inh. |
| 12 [2388-1] | 3'-GTTGTACGTACCTTCTG-5' | ~65% spec. inh. |
| 13 [2525-1] | 3'-TGTTGTACGTACCTTCTG-5' | ~70% spec. inh. |
| 14 [2498-1] | 3'-CCTGTTGTACGTACCTTCTG-5' | ~70% spec. inh. |

| SEQ. ID NO. | SEQUENCE | INHIBITION |
|---|---|---|
| 15 [2492-1] | 3'-**TAC**GTACCTTCTGAACAAT-5' | ~80% spec. inh. |
| 16 [2589-1] | 3'-CGTACCTTCTGAACAAT-5' | ~90% spec. inh. |
| 17 [2590-1] | 3'-**TAC**GTACCTTCTGAACA-5' | ~90% spec. inh. |

TABLE II

ANTISENSE OLIGOMERS TARGETED TO THE TRANSLATION INITIATION CODON OF HPV-11 E7

| SEQ. ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 18 [2644-1] | 3'-CUGUUG **UAC**GU ACCUUCUG-5' | 2'OMeRNA |
| 19 [2624-1] | 3'-CTGTTG **TAG**GT ACCTTCTG-5' | Racemic MP |
| 20 [2571-1] | 3'-CTGTTG **TAC**GT ACCTTCTG-5' | 75%Rp-Mp |
| | | |
| 21 [3130-2,3] | 3'-CCTGTTG **TAC**GT ACCTTCTG-5' | Rp-MP/DE |
| | | |
| 14 [2498-1] | 3'-CCTGTTG **TAC**GT ACCTTCTG-5' | (DE) |
| 22 [2566-1] | 3'-CCTGTTG **TAC**GT ACCTTCTG-5' | (PS) |
| | | |
| 24 [2682-1] | 3'-CCUGUUG(**TAC**GT)ACCUUCUG-5' | [2'OMe] [DE] [2'OMe] |
| 25 [2683-1] | 3'-CCUGUUG(**TAC**GT)ACCUUCUG-5' | [2'OMe] [PS] [2'OMe] |
| | | |
| 26 [2567-1] | 3'-CCTGTTG(**TAC**GT)ACCTTCTG-5' | [MP] [DE] [MP] |
| 27 [2681-1] | 3'-CCTGTTG(**TAC**GT)ACCTTCTG-5' | [MP] [PS/DE] [MP] |
| 28 [2687-1] | 3'-CCTGTTG(**TAC**GT)ACCTTCTG-5' | [75%RpMP] [DE] [75%RpMP] |
| | | |

| SEQ. ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 29 [3169-1,2] | 3'-CCTGTTG(**TAC**GT)ACCTTCTG-5' | [Rp-MP/DE] $[DE]_5$ [Rp-MP/DE] |
| 30 [3214-1] | 3'-CCTGTTG(**TAC**GT)ACCTTCTG-5' | [Rp-MP/DE] $[PS/DE]_7$ [Rp-MP/DE] |
| 31 [3257-1] | 3'-CCTGTTG(**TAC**GTAC)CTTCTG-5' | [Rp-MP/DE] $[PS/DE]_7$ [Rp-MP/DE] |
| 32 [3256-1] | 3'-CCTGTTG(**TAC**GT)ACCTTCTG-5' | [Rp-MP/DE] $[PS]_5$ [Rp-MP/DE] |
| | | |
| 33 [3341-1] | 3'-CCUGUUG(**TAC**GTAC)CUUGUG-5' | 2'-OMe [Rp-MP/DE] $[PS]_7$ 2'-O-Me [Rp-MP/DE] |
| 34 [3336-1] | 3'-CCUGUUG(**TAC**GTAC)CUUCUG-5' | 2'-OMe [Rp-MP/DE] $[PS/DE]_7$ 2'-O-Me [Rp-MP/DE] |
| 35 [2709-1] | 3'AACAATGGGATTTCCTATA-5' | 75%Rp-MP |
| 36 [2588] | 3'-**UAC**GUACCUUCUGAACAAU-5' | 2'-OMe RNA |
| 37 [2740] | 3'AACAAUGGGAUUUCCUAUA-5' | 2'-OMe RNA |
| 77 [3327] | 3'CCTCTTG(**TAC**GTAC)CTTCTG-5' | [MP/2'-O-MeDE] $[PS]_7$ [MP/2'-O-MeDE] |

TABLE III

OLIGOMERS TARGETED TO THE TRANSLATION INITIATION CODON OF HPV-11 E1

A. Sequence comparison around the E1 translation initiation codon and splice donor site at nucleotide 847 (N820-870):

| SEQ. ID. NO. | |
|---|---|
| 38 [HPV-6b] | GA-C------C----------T--- |
| 39 [HPV-11] | ACCATAACAAGGATGGCGGACGATTCAGGTACAGAAAATGAGGGGTCGGGG |

B. Antisense oligomers

| SEQ. ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 40 [2555-1] | 3'-CTACCGCCTGCTAAGTCC-5' | DE |
| 41 [2556-1] | 3'-GCCTGCTAAGTCCATGTC-5' | DE |
| 42 [2557-1] | 3'-GTCCATGTCTTTTACTCCCC-5' | DE |
| | | |
| 43 [3105-1] | 3'-CUACCGCCUGCUAAGUC-5' | 2'OMeRNA |
| 44 [3106-1] | CAUGUCUUUUACUCCCCAG-5' | 2'OMeRNA |
| | | |
| 45 [3196-1] | 3'-CTACCGCCTGCTAAGTCCATG-5' | Rp-MP/DE |

| SEQ. ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 46 [3218-1] | 3'-CTACCGCCTGCTAAGTCCATG-5' | [Rp-MP/DE] [PS/DE] [Rp-MP/DE] |
| | | |
| 47 [2744-1] | 3'-GUCCAUG(TCTTT)UACUCCCC-5' | 2'OMe-DE-2'-OMe |

TABLE IV

OLIGOMERS TARGETED TO THE TRANSLATION INITIATION CODON OF HPV-11 E2

A. Sequence comparison around the E2 translation initiation codon (N2705-2749):

| SEQ. ID NO. | |
|---|---|
| 48 [HPV-6b] | ATTCTGAGGACGAGGAAG**ATG**GAAGCAATAGCCAAGCGTTTAGAT |
| 49 [HPV-11] | ----A---------- |

B. Antisense oligomers

| SEQ ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 50 [2167-1] | CTGCTCCTTC**TAC**CTTCGTT | PS |
| 51 [2550-1] | CTGCTCCTTC**TAC**CTTCG | DE |
| 52 [3102-1] | CCTTC**TAC**CTTCGTTATCGG | DE |
| 53 [3103-1] | C**TAC**CTTCGTTATCGGTTCG | DE |
| | | |
| 54 [2699-1] | CCUGCUCCUUC**UAC**CUUCG | 2'OMeRNA |
| 64 [3129-1] | CCUUC**UAC**CUUCUUAUCGG | 2'OMeRNA |
| 55 [3173-1] | C**UAC**CUUCGUUAUCGGUUCG | 2'OMeRNA |
| | | |

| SEQ ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 56 [3123-1] | TCCTGCTCCTTC**TAC**CTTCG | Rp-MP/DE |
| 57 [3124-1] | CCTTC**TAC**CTTCGTTATCGGTTCG | Rp-MP/DE |
| 58 [3125-1] | C**TAC**CTTCTTATCGGTTCG | Rp-MP/DE |
| | | |
| 59 [3170-1] | TCCTGCT(CCTTC)**TAC**CTTCG | [Rp-MP/DE] $[DE]_5$ [Rp-MP/DE] |
| 60 [3233-1] | CTTC**TAC**C(TTCGT)TATCGGTTC | [Rp-MP/DE] $[DE]_5$ [Rp-MP/DE] |
| 61 [3234-1] | CTTC**TAC**C(TTCGT)TATCGGTTC | [Rp-MP/DE] $[PS/DE]_5$ [Rp-MP/DE] |
| | | |
| 62 [2625-1] | CCTGCTCCTTC**TAC**CTTCG | Racemic MP |
| 63 [2574-1] | CCTGCTCCTTC**TAC**CTTCG | 75%Rp-MP |

TABLE V

OLIGOMERS TARGETED TO THE TRANSLATION INITIATION CODON OF HPV-11 E6

A. Sequence comparison around the E6 translation initiation codon:

| SEQ. ID NO. | |
|---|---|
| 65 [HPV-6b(N90-142)] | -A----------GC-A----------GA-C----- |
| 66 [HPV-11(N90-142)] | AGACGAGGCATTATGGAAAGTAAAGATGCCTCCACGTCTGCAACATCTATAGA |

B. Antisense oligomers

| SEQ. ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 67 [2702-1] | TGCTCCGTAATACCTTTCA | DE |
| 68 [2701-1] | TAATACCTTTCATTTCTA | DE |
| 69 [2703-1] | TAATACCTTTCATTTCTACGGAGG | DE |
| | | |
| 70 [2802-1] | UGCUCCGUAAUACCUUUCA | 2'OMeRNA |
| 71 [2803-1] | UUUCUACGGAGGUGCAGA | 2'OMeRNA |
| | | |
| 72 [3161-1,2,3] | CTGCTCCGTAATACCTTTCA | Rp-MP/DE |
| | | |

| SEQ. ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 73 [3219-1] | CUGCUCC(GTAAT)ACCUUUCA | [2'OMeRNA] [PS] [2'OMeRNA] |
| 74 [3220-1] | CUGCUCC(GTAAT)ACCUUUCA | [2'OMeRNA] [DE] [2'OMeRNA] |
| | | |
| 75 [3255-1] | CTGCTCC(GTAAT)ACCTTTCA | [Rp-MP/DE] [DE] [Rp-MP/DE] |
| 76 [3215-1] | CTGCTCC(GTAAT)ACCTTTCA | [Rp-MP/DE] [PS/DE] [Rp-MP/DE] |

TABLE VI

POTENCY OF OLIGOMERS TARGETED TO HPV-11 E7

| SEQ. ID NO. | BACKBONE | Tm | Cell-free assay | | | Vero cells Microinjection |
|---|---|---|---|---|---|---|
| | | | IC50 | IC90 | CAT Inhibition | |
| 7 [2552-1] | All-DE | | >1 μM | >>1 μM | 40%, 1 μM | N/D |
| 11 [2553-1] | All-DE | | 0.03 μM | 0.2 μM | 35%, 1 μM | N/D |
| 12 [2388-1] | All-DE | | 0.03 μM | 0.2 μM | 20%, 1 μM | N/D |
| 15 [2492-1] | All-DE | | 0.005 μM | 0.05 μM | 20%, 0.1 μM | N/D |
| 14 [2498-1] | All-DE | | ~0.1 μM | >1 μM | 45%, 1 μM | N/D |
| 22 [2566-1] | All-PS | | ~0.15 μM | 0.5 μM | 60%, 0.4 μM | N-3+ (0.5 μM) |
| 26 [2567-1] | [MP][DE][MP] | 45.6 | ~0.2 μM | >>1 μM | No inh., 1 μM | N-2+ (0.5 μM) C-3+ (10 μM) |
| 28 [2687-1] | [75%Rp-MP][DE][75%Rp-MP] | 52.8 | ~0.04 μM | 1 μM | 20%, 5 μM | N-3+ (0.5 μM) |
| 29 [3169-1] | [Rp-MP/DE][DE]$_5$[Rp-MP/DE] | 62.6 | ~0.04 μM | 0.8 μM | 20%, 5 μM | C-3+ (2 μM) |
| 30 [3214-1] | [Rp-MP/DE][DE/PS]$_5$[Rp-MP/DE] | 61.0 | ~0.2 μM | 4 μM | No inh., 10 μM; 25%, 5 μM | C-3+ (1 μM) |
| 31 [3257-1] | [Rp-MP/DE][DE/PS]$_7$[Rp-MP/DE] | 60.9 | ~0.06 μM | 0.6 μM | 50%, 2 μM | C-3+ (0.5 μM) |
| 32 [3256-1] | [Rp-MP/DE][PS]$_5$[Rp-MP/DE] | 60.1 | ~0.25 μM | 5 μM | No inh., 5 μM | C-3+ (0.5 μM) |
| 34 [3336-1] | [Rp-MP/DE][DE/PS]$_7$[Rp-MP/DE] | 66.8 | ~0.3 μM | >>10 μM | No inh., 5 μM | N/D |
| 33 [3341-1] | [Rp-MP/DE][PS]$_7$[Rp-MP/DE] | 65.8 | 2 μM | >>10 μM | 30%, 5 μM | N/D |
| 24 [2682-1] | [2'OMeRNA][DE]$_5$[2'OMeRNA] | 72.5 | ~0.015 μM | 0.4 μM | 15%, 1 μM | N-2+ (0.5 μM) C-3+ (10 μM) |

| SEQ. ID NO. | BACKBONE | Tm | Cell-free assay | | | Vero cells Microinjection |
|---|---|---|---|---|---|---|
| | | | IC50 | IC90 | CAT Inhibition | |
| 25 [2683-1] | [2'OMeRNA][PS]s[2'OMeRNA] | | N/D | | | N-3+ (0.5 μM) C-3+ (10 μM) |

TABLE VII

POTENCY OF OLIGOMERS TARGETED TO HPV-11 E7 IN A CELL BASED ASSAY

| | | Cell-based assay | |
|---|---|---|---|
| SEQ.ID.NO. | Backbone | IC50 | IC90 |
| 29[3169-2] | [Rp-MP/DE] - $[DE]_5$[Rp-MP/DE] | >2 uM | >>10 uM |
| 30[3214-1] | [Rp-MP/DE] - $[DE/PS]_5$[Rp-MP/DE] | 0.2 uM | 1 uM |
| 32[3256-1] | [Rp-MP/DE] - $[PS]_5$[Rp-MP/DE] | 0.12 uM | 1 uM |
| 31[3257-1] | [Rp-MP/DE] - $[DE/PS]_7$[Rp-MP/DE] | 0.06 uM | <0.3 uM |
| 33[3341-1] | 2'OMe[Rp-MP/DE] - $[PS]_7$2'OMe[Rp-MP/DE] | ND | ND |
| 34[3336-1] | 2'OMe[Rp-MP/DE] - $[DE/PS]_7$2'OMe[Rp-MP/DE] | 0.4 uM | ~2 uM |
| 77[3327-1] | [MP/2'OMeDE] $[PS]_7$[MP/2'0MeDE] | 0.5 $\mu$M | ~2 $\mu$M |

TABLE VIII

POTENCY OF OLIGOMERS TARGETED TO HPV-11 E7 IN MICROINJECTION IN VERO CELLS

| E7 OLIGOMERS | | | Oligo Concentration of Microinjection Solution | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Nuclear Injection | | | | | Cytoplasmic Injection | | | | |
| SEQ.ID.NO | Target | Backbone | 4+ | 3+ | 2+ | 1+ | 0 | 4+ | 3+ | 2+ | 1+ | 0 |
| 22[2566] | 11/E7,AUG/-7 | PS | | 0.5μM | | | | | | | | 0.5μM |
| 26[2567] | 11/E7,AUG/-7 | MP/DE/MP | | | 0.5μM | | | | 10μM* | | | 0.5μM |
| 28[2687] | 11/E7,AUG/-7 | 75%RpMP/DE/75%RpMP | | 0.5μM | | | | | | | | 0.5μM |
| 24[2682] | 11/E7,AUG/-7 | 2'OMe/DE/2'OMe | | | 0.5μM | | | | 10μM | | | 0.5μM |
| 25[2683] | 11/E7,AUG/-7 | 2'OMe/PS/2'OMe | | 0.5μM | | | | | 10μM* | | | 0.5μM |
| 29[3169-1] | 11/E7,AUG/-7 | [RpMP/DE]-[DE]-[RpMP/DE] | | | | | | 5μM | 2μM* | 0.5μM | | |
| 30[3214] | 11/E7,AUG/-7 | [RpMP/DE]-[PS/DE]*[RpzMP/DE] | | | | | | 2μM | 1μM | 0.5μM | | |
| 31[3257] | 11/E7,AUG/-7 | [RpMP/DE]-[PS/DE]'[RpMP/DE] | | | | | | 2μM | 0.5μM | | | |
| 32[3256] | 11/E7,AUG/-7 | [RpMP/DE]-[PS]*[RpMP/DE] | | | | | | 2μM | 0.5μM | | | |
| 18[2644] | 11/E7,AUG/-6 | 2'OMeRNA | | | | | 1μM | 10μM* | | | | |
| 20[2571] | 11/E7,AUG/-6 | 75%RpMP | | | | | | | | 10μM | | 0.5μM |
| 35[2709] | 11/E7,AUG/+15 | 75%RpMP | | | | | | | | | 0.5μM | |
| 2571+2709 | | | | | | | | | | | 0.5μM each | |
| 36[2588] | 11/E7,AUG/+1 | 2'OMeRNA | | | | | 1μM | | | 10μM* | | 1μM |
| 37[2740] | 11/E7,AUG/+15 | 2'OMeRNA | | | | | 1μM | | | | 10μM | 1μM |
| 21[3130-2] | 11/E7,AUG/-7 | RpMP/DE | | | | | | | | | 10μM* | |

TABLE IX

POTENCY OF OLIGOMERS TARGETED TO HPV-11 E1 IN CELL FREE ASSAY

A.

| | | | Cell-free assay | | |
|---|---|---|---|---|---|
| SEQ. ID. NO. | Target | Backbone | IC50 | IC90 | CAT Inhibition |
| 45 [3196-1] | AUG+1 | Rp-MP/DE | >10 μM | >>10 μM | No inh. (10 μM) |

B.

| SEQ. ID NO. | Backbone | Sequence | % inhibition of E1 (activity at 1 μM | % inhibition of CAT (specificity at 1 μM) |
|---|---|---|---|---|
| 40 [2551-1] | Phosphodiester | CTACCGCCTGCTAAGTCC | 90 | 72 |
| 43 [3105-1] | 2'OMeRNA | CUACCGCCUGCUAAGUC | 50 | 10 |
| | | | | |
| 41 [2556-1] | Phosphodiester | GCCTGCTAAGTCCATGTC | 92 | 80 |
| | | | | |
| 42 [2557-1] | Phosphodiester | GTCCATGTCTTTTACTCCCC | 98 | 25 |
| 47 [2744-1] | 2'OMe-DE-2'OMe | GTUCCAUG(TCTTT)UACUCCCC | 100 | 0 |

TABLE X

POTENCY OF OLIGOMERS TARGETED TO HPV-11 E2 IN CELL FREE ASSAY

| | | | Cell-free assay | | |
|---|---|---|---|---|---|
| SEQ. ID NO. | Target | Backbone | IC50 | IC90 | CAT-inhibition |
| 57 [3124-1] | AUG-5 | Rp-MP/DE | 5 μM | >>10 μM | 10% (5 μM) |
| 59 [3170-1] | AUG-12 | [Rp-MP/DE] $[DE]_5$ [Rp-MP/DE] | ~0.06 μM | ~1 μM | 20% (5 μM) |
| 60 [3233-1] | AUG-4 | [Rp-MP/DE] $[DE]_5$ [Rp-MP/DE] | ~0.1 μM | ~1 μM | 20% (5 μM) |
| 61 [3234-1] | AUG-4 | [Rp-MP/DE] $[DE/PS]_5$ [Rp-MP/DE] | ~0.1 μM | ~1 μM | 15% (10 μM) |

TABLE XI

ANTISENSE ACTIVITY OF OLIGONUCLEOTIDES TARGETED TO THE TRANSLATION INITIATION CODON OF HPV-11 E2

| SEQ. ID NO. | Target | Backbone | Sequence | TM | % inhibition of E2 (activity at 1 μM) | % inhibition of CAT (activity at 1 μM) |
|---|---|---|---|---|---|---|
| 50 [2167-2] | E2, AUG-10 | Phosphorothioate | CTGCTCCTTCTACCTTCGTT | 59.6 | | |
| 51 [2550-1] | E2, AUG-10 | Phosphodiester | CTGCTCCTTCTACCTTCG | | 55 | 15 |
| 54 [2699-1] | E2, AUG-11 | 2'OMeRNA | UCCUGCUCCUUCUACCUUCG | | 70 | 0 |
| 62 [2625-1] | E2, AUG-11 | Racemic MP | CCTGCTCCTTCTACCTTCG | 42.9 | 0 | 0 |
| 63 [2574-1] | E2, AUG-11 | 75% Rp-MP | CCTGCTCCTTCTACCTTCG | 51.8 | 0 | 0 |
| 56 [3123-1] | E2, AUG-12 | Rp-MP/DE | TCCTGCTCCTTCTACCTTCG | 68.4 | 0 | 0 |
| | | | | | | |
| 52 [3102-1] | E2, AUG-5 | Phosphodiester | CCTTCTACCTTCGTTATCGG | | 80 | 25 |
| 64 [3129-1] | E2, AUG-5 | 2'OMeRNA | CCUUCUACCUUCGUUAUCGG | | 35 | 0 |
| 57 [3124-1] | E2, AUG-5 | Rp-MP/DE | CCTTCTACCTTCGTTATCGGTTCG | 66.4 | 35 | 0 |
| | | | | | | |
| 53 [3103-1] | E2, AUG-1 | Phosphodiester | CTACCTTCGTTATCGGTTCG | | 100 | 80 |
| 55 [3173-1] | E2, AUG-1 | 2'OMeRNA | CUACCUUCGUUAUCGGUUCG | 81.1 | 40 | 0 |

TABLE XII

POTENCY OF OLIGOMERS TARGETED TO HPV-11 E2 IN MICROINJECTION TO VERO CELLS

| Microinjection Summary | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E2 OLIGOMERS | | | Oligo Concentration of Microinjection Solution | | | | | | | | | | |
| | | | Nuclear Injection | | | | | Cytoplasmic Injection | | | | | |
| SEQ.ID.NO | Target | Backbone | 4+ | 3+ | 2+ | 1+ | 0 | 4+ | 3+ | 2+ | 1+ | 0 |
| 60[3233] | 11/E2,AUG/-4 | [RpMP/DE]-[DE]$_8$[RpMP/DE] | | | | | | 2μM | 1μM | 0.5μM | | |
| 61[3234] | 11/E2,AUG/-4 | [RpMP/DE]-[PS/DE]$_8$[RpMP/DE] | | | | | | 1μM | 0.5μM | | | |
| 59[3170] | 11/E2,AUG/-12 | [RpMP/DE]-[DE]$_8$[RpMP/DE] | | | | | | 2μM | 1μM | 0.5μM | | |
| 63[2574-1] | 11/E2,AUG/-10 | 75%RpMP | | | | | | 2μM* | 1μM | 0.5μM | | |
| 54[2699-1] | 11/E2,AUG/-10 | 2'MeRNA | | | | | | 2μM | | | | |
| 56[3123-1] | 11/E2,AUG/-12 | RpMP/DE | | | | | | | | | 10μM | 2μM |
| 57[3124-1] | 11/E2,AUG/-5 | RpMP/DE | | | | | | | 2μM<br>10μM* | | | |
| 58[3125-2] | 11/E2,AUG/-1 | RpMP/DE | | | | | | 10μM | 2μM<br>5μM | 1μM | 0.5μM | |

TABLE XIII

POTENCY OF OLIGOMERS TARGETED TO HPV-11 E6 IN CELL FREE ASSAY OF OLIGONUCLEOTIDES

TABLE A

| SEQ.ID.NO. | Backbone | Sequence | % inhibition of E6 (activity at 1μM) | % inhibition of CAT (specificity at 1μM) | TM |
|---|---|---|---|---|---|
| 68[2701-1] | Phosphodiester | TAATACCTTTCATTTCTA | 5 | 0 | |
| 69[2703-1] | Phosphodiester | TAATACCTTTCATTTCTACGGAGG | 35 | 15 | |
| | | | | | |
| 67[2702-1] | Phosphodiester | TGCTCCGTAATACCTTTCA | 30 | 5 | |
| 70[2802-1] | 2'OMeRNA | UGCUCCGUAAUACCUUUCA | 65 | 0 | 75.7 |
| 72[3161-1] | Rp-MP/DE | CTGCTCCGTAATACCTTTCA | 55 | 0 | 60.6 |

TABLE B

| | | | Cell-free assay | | |
|---|---|---|---|---|---|
| SEQ.ID.NO. | Target | Backbone | IC50 | IC90 | CAT inhibition |
| 72[3161-1] | AUG-10 | Rp-MP/DE | 1 uM | >>10 uM | 10% (10uM) |
| 75[3255-1] | AUG-10 | [Rp-MP/DE][DE]$^5$[Rp-MP/DE] | 1 uM | 5 uM | No inh. (10 uM) |
| 76[3215-1] | AUG-10 | [Rp-MP/DE][DE/PS]$^5$[Rp-MP/DE] | 0.3 uM | 2 uM | no inh. (10 uM) |

TABLE XIV

| SEQ. ID. NO. | Backbone | Oligo concentration (μM) 0.01 | 0.1 | 1 | 10 |
|---|---|---|---|---|---|
| 26 [2567-1] | [MP]-[DE]$_5$-[MP] | 2 | 15 | 50 | 80 |
| 29 [3169-1] | [Rp-MP/DE]-[DE]$_5$[Rp-MP/DE] | 7 | 45 | 85 | 100 |
| 30 [3214-1] | [Rp-MP/DE]-[PS/DE]$_5$-[Rp-MP/DE] | 2 | 20 | 50 | 80 |
| 31 [3257-1] | [Rp-MP/DE]-[PS/DE]$_7$-[RP-MP/DE] | 4 | 40 | 75 | 100 |
| 33 [3341-1] | 2'OMe[Rp-MP/DE]-[PS]$_7$-2'OMe[Rp-MP/DE] | 5 | 40 | 60 | 60 |
| 34 [3336-1] | 2'OMe[Rp-MP/DE]-[PS/DE]$_7$-2'OMe[Rp-MP/DE] | 5 | 50 | 60 | 65 |

Results are percentage of cleavage of E7 mRNA. Estimated values were obtained by visual inspection of the gel.

TABLE XV

ANTISENSE OLIGOMERS TARGETED TO THE TRANSLATION INITIATION CODON OF HPV-16 E7

| SEQ. ID NO. | SEQUENCE | BACKBONE |
|---|---|---|
| 99 [3678-1] | 5'-GCATGA(TTACA)GCTGGGTTT-3' | [Rp-MP/DE] $[PS]_8$ [Rp-MP/DE] |
| 100 [3679-1] | 5'-CCATGCAT(GATTA)CAGCTGG-3' | [Rp-MP/DE] $[PS]_8$ [Rp-MP/DE] |
| 101 [3680-1] | 5'-GTATCTCC(ATGCA)TGATTACAG-3' | [Rp-MP/DE] $[PS]_8$ [Rp-MP/DE] |
| Control 102 [3268] | 5'-CCTCATTTT(ATTAG)GAAAGGACAG-3' | [Rp-MP/DE] $[PS]_8$ [Rp-MP/DE] |

Claims

1. An oligomer having either at least one internucleosidyl linkage which is not a phosphodiester linkage or at least one nucleosidyl unit which has a 2'-substituted ribosyl moiety and which has from about 14 to about 35 nucleosidyl units and which has a nucleoside base sequence complementary to a target region of an mRNA or pre-mRNA (a) which target region includes a sequence selected from a translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a polyadenylation signal, a 3'-untranslated region and a 5'-untranslated region and (b) wherein the mRNA or pre-mRNA codes for a human papilloma virus gene selected from E1, E2, E6 and E7.

2. An oligomer according to claim 1 which is capable of decreasing expression of the virus gene by at least about 75 percent.

3. An oligomer according to claim 2 wherein said gene is E6 or E7.

4. An oligomer according to claim 2 wherein said target region is about -20 to +20 nucleosides of the splice donor site, about -20 to about +20 nucleosides of the splice acceptor site or about -25 to about +35 nucleotides of the initiation codon.

5. An oligomer according to claim 4 wherein said HPV is HPV-6b or HPV-11.

6. An oligomer according to claim 5 wherein said gene is E6 or E7.

7. An oligomer according to claim 4 wherein said gene is E6 or E7.

8. An oligomer according to claim 2 comprising an RNase H-activating region and a non-RNase H activating region, wherein

the RNase H-activating region comprises a segment of at least three consecutive 2'-unsubstituted nucleosides linked by charged internucleoside linkage structures,

the non-RNase H-activating region comprises a segment of at least two linked nucleosides, at least one of the linkages in said non-RNase H-activating region being chirally-selected.

9. An oligomer according to claim 8 wherein said RHase H-activating region comprises between five and about nine consecutive linked nucleosides.

10. An oligomer according to claim 9 wherein the charged linkage structures in said RNase H-activating region are selected from the group consisting of phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages.

11. An oligomer according to claim 10 wherein the segment of charged linkage structures in said RNase H-activating region comprises a mixed charged linkage sequence including at least two different charged linkage structures.

12. An oligomer according to claim 11 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

13. An oligomer according to claim 2 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

14. An oligomer according to claim 13 which has from about 18 to about 24 nucleosidyl units.

15. An oligomer according to claim 11 which has the nucleoside sequence 3'-CCTGTTGTACGTACCTTCTG-5' [SEQ. ID. NO. 96].

16. An oligomer according to claim 1 comprising an RNase H-activating region and a non-RNase H activating region, wherein

the RNase H-activating region comprises a segment of at least three consecutive 2'-unsubstituted nucleosides linked by charged internucleoside linkage structures,

the non-RNase H-activating region comprises a segment of at least two linked nucleosides, at least one of the linkages in said non-RNase H-activating region being chirally-selected.

17. An oligomer according to claim 16 wherein said RHase H-activating region comprises between five and about nine consecutive linked nucleosides.

18. An oligomer according to claim 17 wherein the charged linkage structures in said RNase H-activating region are selected from the group consisting of phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages.

19. An oligomer according to claim 18 wherein the segment of charged linkage structures in said RNase H-activating region comprises a mixed charged linkage sequence including at least two different charged linkage structures.

20. An Oligomer according to claim 1 having phosphonate internucleosidyl linkages selected from the

group consisting of lower alkylphosphonate internucleosidyl linkages of 1 to 3 carbon atoms and lower alkylphosphonothioate internucleosidyl linkages of 1 to 3 carbon atoms which are mixed with non-phosphonate internucleosidyl linkages wherein the phosphonate linkages are interspersed between single non-phosphonate internucleosidyl linkages in a ratio of from about 1 to 1 to about 1 to 4 non-phosphonate linkages to phosphonate linkages and wherein the Oligomer is substantially complementary to the RNA target sequence.

21. An oligomer according to claim 20 wherein said phosphonate linkages are chirally pure Rp methylphosphonate linkages.

22. An oligomer according to claim 21 wherein said non-phosphonate linkages are selected from the group consisting of phosphodiester, phosphotriester, phosphorothioate, phosphorodithioate, phosphoramidate, phosphorofluoridate, boranophosphate, formacetal and silyl.

23. An oligomer according to claim 22 wherein the nucleosides of said oligomer have 2'-O-methyl ribosyl groups as sugar moieties.

24. An oligomer complementary to an mRNA or pre-mRNA encoding E7 of an HPV which comprises about 14 to about 35 nucleosidyl units and which has a nucleoside base sequence which is complementary to a portion of the mRNA or pre-mRNA in the region of -25 to +35 wherein +1 to +3 is the initiation codon.

25. An oligomer which has a nucleoside base sequence complementary to an mRNA or pre-mRNA coding for E6 or E7 of an HPV having a pseudoknot region wherein

the oligomer has from about 14 to about 35 nucleosidyl units and is complementary to a target sequence of the mRNA or pre-mRNA immediately 5- or immediately 3'- to the pseudoknot region.

26. An oligomer according to claim 25 wherein said target sequence is immediately 3'- to the pseudoknot region.

27. An oligomer according to claim 26 wherein the pseudoknot region has a sequence selected from
5'-AGGCAGCUUCCCAGUGGAAGGGUCGUUGCUUCUGCU-3'
or
5'-AGGCGCGGUUCGUGGAAGGGUCGCUGCCUCUGCU-3' [SEQ. ID NOS. 97 and 98].

28. An oligomer which has from about 14 to about 35 nucleosidyl units and which has a nucleoside base sequence complementary to a target region of an mRNA or pre-mRNA (a) which target region includes a sequence selected from a translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a polyadenylation signal, a 3'-end of an untranslated region and a 5'-end of an untranslated region and (b) wherein the mRNA or pre-mRNA codes for a human papilloma virus gene selected from E1, E2, E6 and E7.

29. An oligomer according to claim 28 wherein said target region is about -20 to +20 nucleosides of the splice donor site, about -20 to about +20 nucleosides of the splice acceptor site or about -25 to about +35 nucleotides of the initiation codon.

30. An oligomer according to claim 29 wherein said HPV is HPV-6b or HPV-11.

31. An oligomer according to claim 30 wherein said gene is E6 or E7.

32. An oligomer according to claim 29 wherein said gene is E6 or E7.

33. An oligomer according to claim 28 comprising an RNase H-activating region and a non-RNase H activating region, wherein

the RNase H-activating region comprises a segment of at least three consecutive 2'-unsubstituted nucleosides linked by charged internucleoside linkage structures,

the non-RNase H-activating region comprises a segment of at least two linked nucleosides, at least one of the linkages in said non-RNase H-activating region being chirally-selected.

34. An oligomer according to claim 33 wherein said RHase H-activating region comprises between five and about nine consecutive linked nucleosides.

35. An oligomer according to claim 34 wherein the charged linkage structures in said RNase H-activating region are selected from the group consisting of phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages.

36. An oligomer according to claim 35 wherein the segment of charged linkage structures in said RNase H-activating region comprises a mixed charged linkage sequence including at least two different charged linkage structures.

37. An oligomer according to claim 36 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

38. An oligomer according to claim 28 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

39. A oligomer according to claim 28 having phosphonate internucleosidyl linkages selected from the group consisting of lower alkylphosphonate internucleosidyl linkages of 1 to 3 carbon atoms and lower alkylphosphonothioate internucleosidyl linkages of 1 to 3 carbon atoms which are mixed with non-phosphonate internucleosidyl linkages wherein the phosphonate linkages are interspersed between single non-phosphonate internucleosidyl linkages in a ratio of from about 1 to 1 to about 1 to 4 non-phosphonate linkages to phosphonate linkages and wherein the Oligomer is substantially complementary to the RNA target sequence.

40. An oligomer according to claim 39 wherein said phosphonate linkages are chirally pure Rp methylphosphonate linkages.

41. An oligomer according to claim 40 wherein said non-phosphonate linkages are selected from the group consisting of phosphodiester, phosphotriester, phosphorothioate, phosphorodithioate, phosphoramidate, phosphorofluoridate, boranophosphate, formacetal and silyl.

42. An oligomer according to claim 41 wherein the nucleosides of said oligomer have 2'-O-methyl ribosyl groups as sugar moieties.

43. An oligomer according to claim 42 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

44. An oligomer according to claim 39 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

45. An oligomer according to claim 28 which is capable of decreasing expression of the virus gene by at least about 75 percent.

46. An oligomer according to claim 4 wherein said HPV is HPV-16.

47. An oligomer according to claim 46 wherein said gene is E6 or E7.

48. An oligomer according to claim 29 wherein said HPV is HPV-16.

49. An oligomer according to claim 48 wherein said gene is E6 or E7.

50. An oligomer according to claim 34 wherein said HPV is HPV-16.

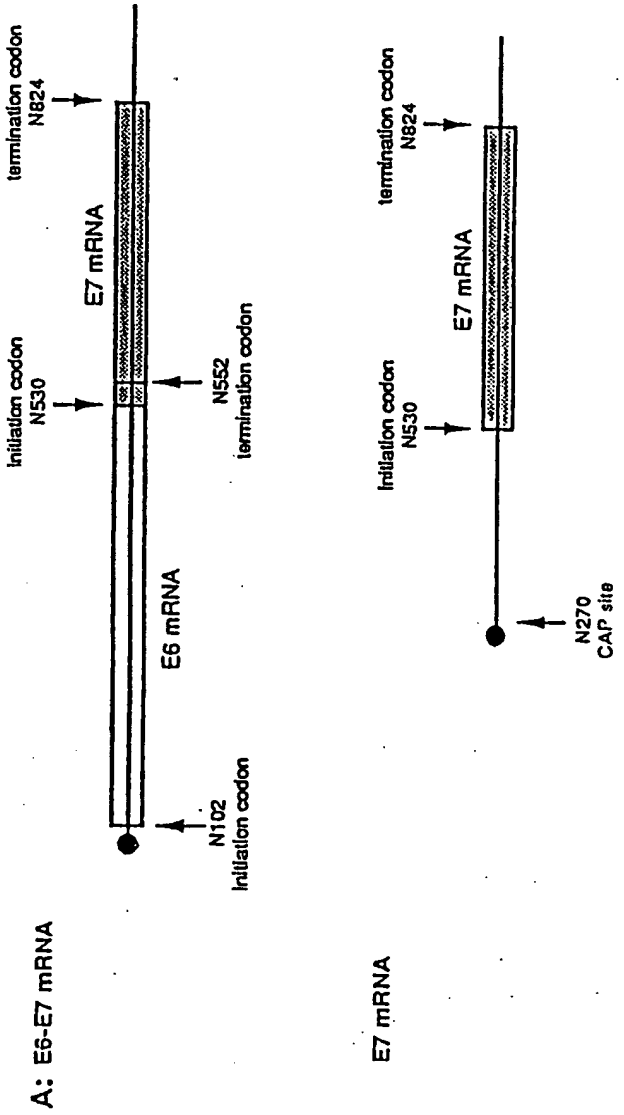
51. An oligomer according to claim 50 wherein said gene is E6 or E7.

52. An oligomer according to claim 39 wherein said HPV is HPV-16.

53. An oligomer according to claim 52 wherein said gene is E6 or E7.

*Figure 1*

*Polycistronic E6/E7 mRNA and monocistronic E7 mRNA found in HPV-6b or HPV-11 condylomas*



# Figure 2 A

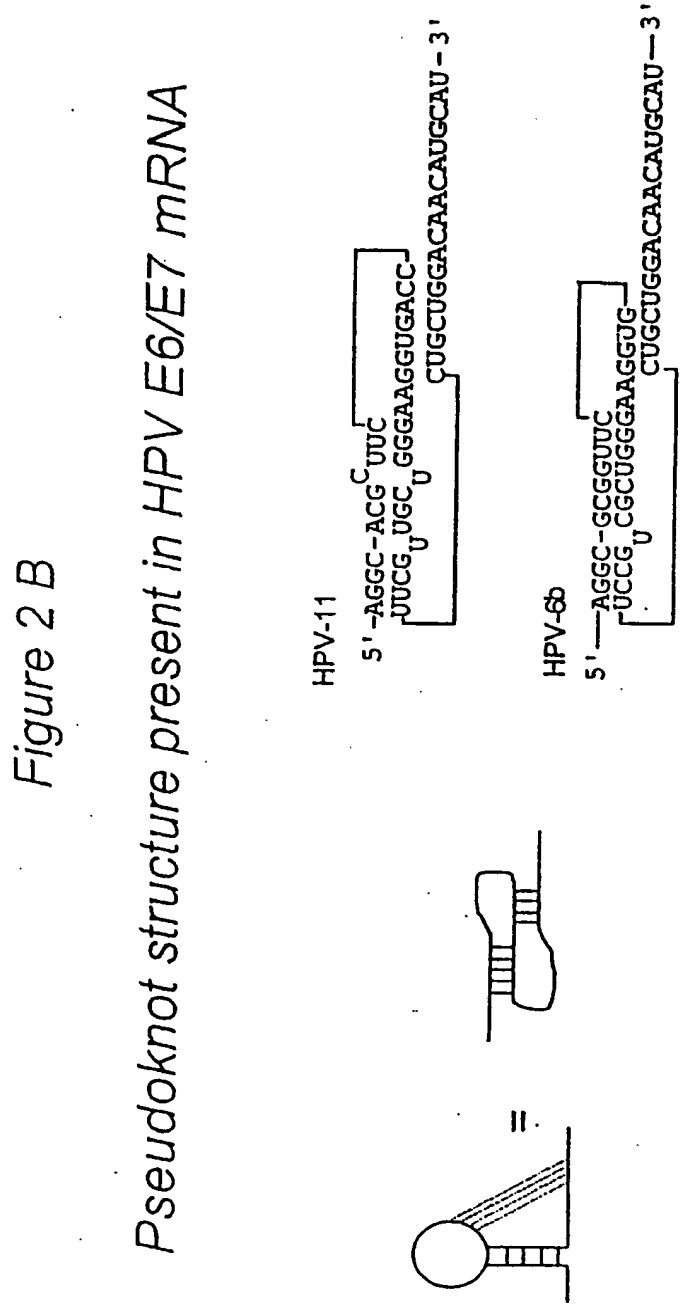
## Aligment of HPV sequences around the E6/E7 boundary

```
            <0--<1-----                  <2----    -----1> --0>      ----2>
           470        480        490         500        510          520              530
HPV 11: GGAAAGGCACGCTTCATAAAACTAAATAA-CCAGTGGAAGGGTCGTTGCTTACA---CTGCTGGAC-----AACATGCAT
HPV 6 : ACCAAGGCGCGGTTCATAAAGCTAAATTG-TACGTGGAAGGGTCGCTGCCTACA---CTGCTGGAC-----AACATGCAT
HPV 44: GACAAGGCGCGATTCATTAAATTACAAGA-TACCTGGAAGGGTCGCTGCTTCCA---TTGTTGGAC-----ATCATGCAT
HPV 43: CAAAAGGCACAATTCTTTAAAATACATAGCGTGGTGGAAAGGATACTGCTTACA---TTGCTGGAA-----ATCATGCAT
cons#1  ...AAGGCRCg.TTC.T.AAa.TA.A..R-....TGGAAgGGtcgcTGCtTaCA---YTGcTGGAc-----A.CATGCAT

                <1---                <2----    ---1>             ----2>
HPV 35: GAAAAAAAACGATTCCATAACATCGGTGG-ACGGTGGACAGGTCGGTGTATGTC---CTGTTGGAA-[27]ATCATGCAT
HPV 16: AAAAAGCAAAGATTCCATAATATAAGGGG-TCGGTGGACCGGTCGATGTATGTC---TTGTTGCAG-[33]ATCATGCAT
HPV 31: AAAAAGAAACGATTCCACAACATAGGAGG-AAGGTGGACAGGACGTTGCATAGC---ATGTTGGAG-[27]AACATGCGT
HPV 33: TTAAACAAACGATTTCATAATATTTCGGG-TCGTTGGGCAGGGCGCTGTGCGGC---GTGTTGGAG-[39]GCCATGAGA
HPV 18: GAAAAACGACGATTTCACAACATAGCTGG-GCACTATAGAGGCCAGTGCCATTC---GTGCTGCAA-[54]AGTATGCAT
HPV 39: AGCAAACGAAGATTTCATAAAATAGCAGG-AAGCTATACAGGACAGTGTCGACG---GTGCTGGAC-[52]GATATGCGT
HPV 51: GAAAAAAAAAAGGTTCCATGAAATAGCGGG-ACGTTGGACGGGGCAATGCGCTAA---TTGCTGGCA-[38]GCCATGCGT
HPV 58: TTAAACAAAAGGTTTCATAATATTTCGGG-TCGTTGGACAGGGCGCTGTGCAGTGTGTTGGAGACCC[39]CCCATGAGA
HPV 2a: GGAAACAAGCGTTTCCACAACATATCAGG-CCGGTGGACGGGACATTGCATGAA---CTGCGGGTC-----ATCATGCAC
cons#2  ..aAA..Ra.GrTTYCAtaA.AT....GG-..R.Tg.acRGG.CR.TGy.....---.TGy.Gr...----..YATG.Ry
```

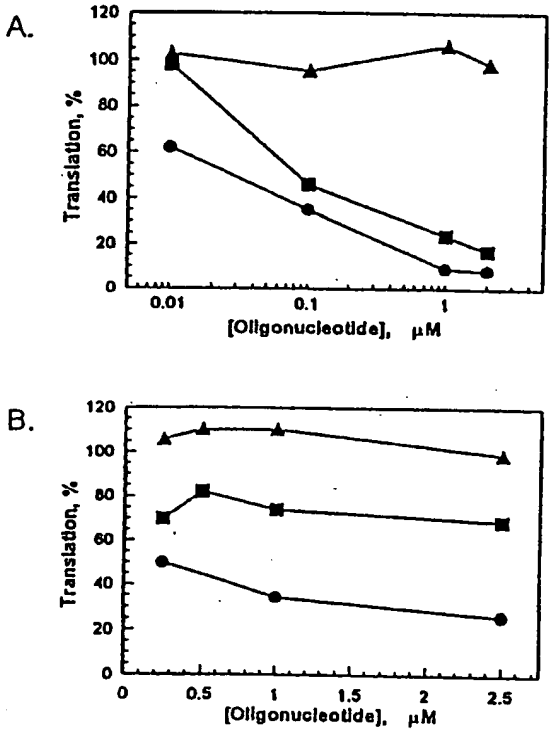
Figure 2 B

Pseudoknot structure present in HPV E6/E7 mRNA



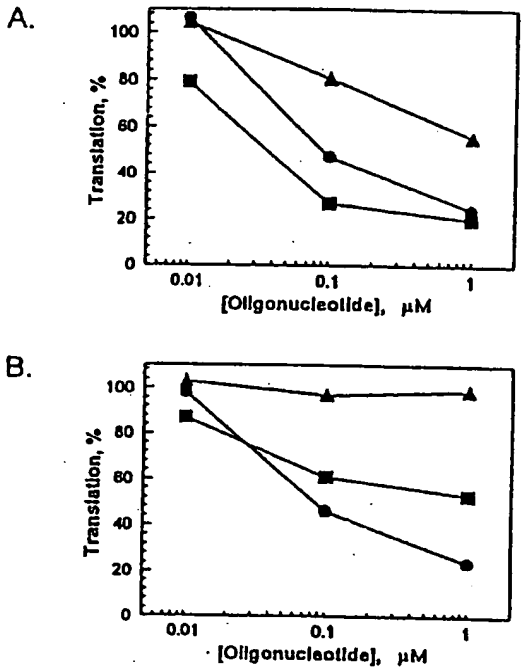
# Figure 3

## Chimeric oligomers inhibit translation of E7 mono and polycistronic mRNA



# Figure 4

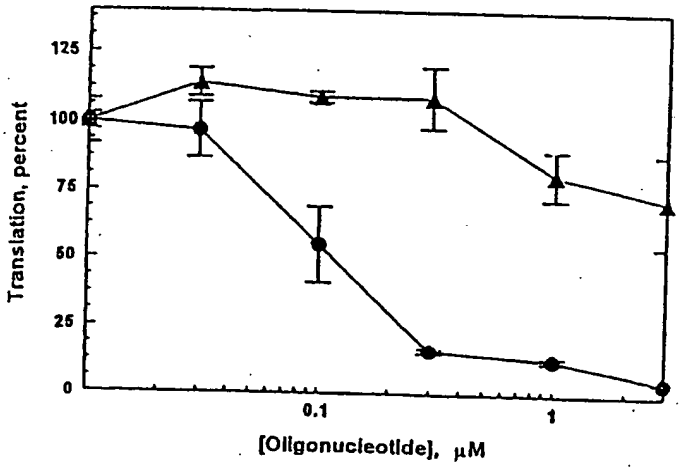
## Inhibition of expression of E6 and E7 proteins by a single oligonucleotide



# Figure 5

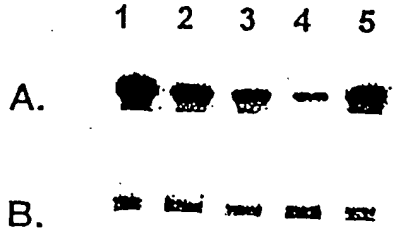
## *Inhibition of HPV-11 E7 protein expression in transiently transfected COS-7 cells*

Dose response with oligomer # 3256, [Rp-MP/DE][PS]$_5$[Rp-MP/DE]



# Figure 6

## Downregulation of E7 mRNA levels in COS-7 cells treated with [Rp-MP/DE][PS][ Rp-MP/DE]



# Figure 7

*Inhibition of expression of E6 and E7 proteins by a single chimeric oligonucleotide*

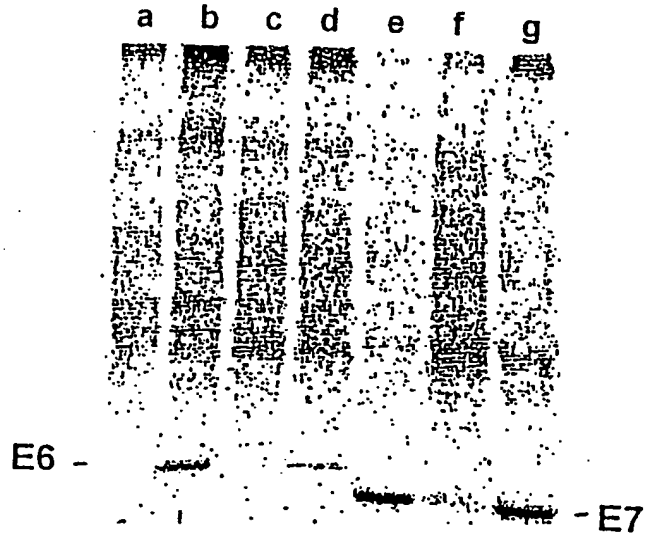
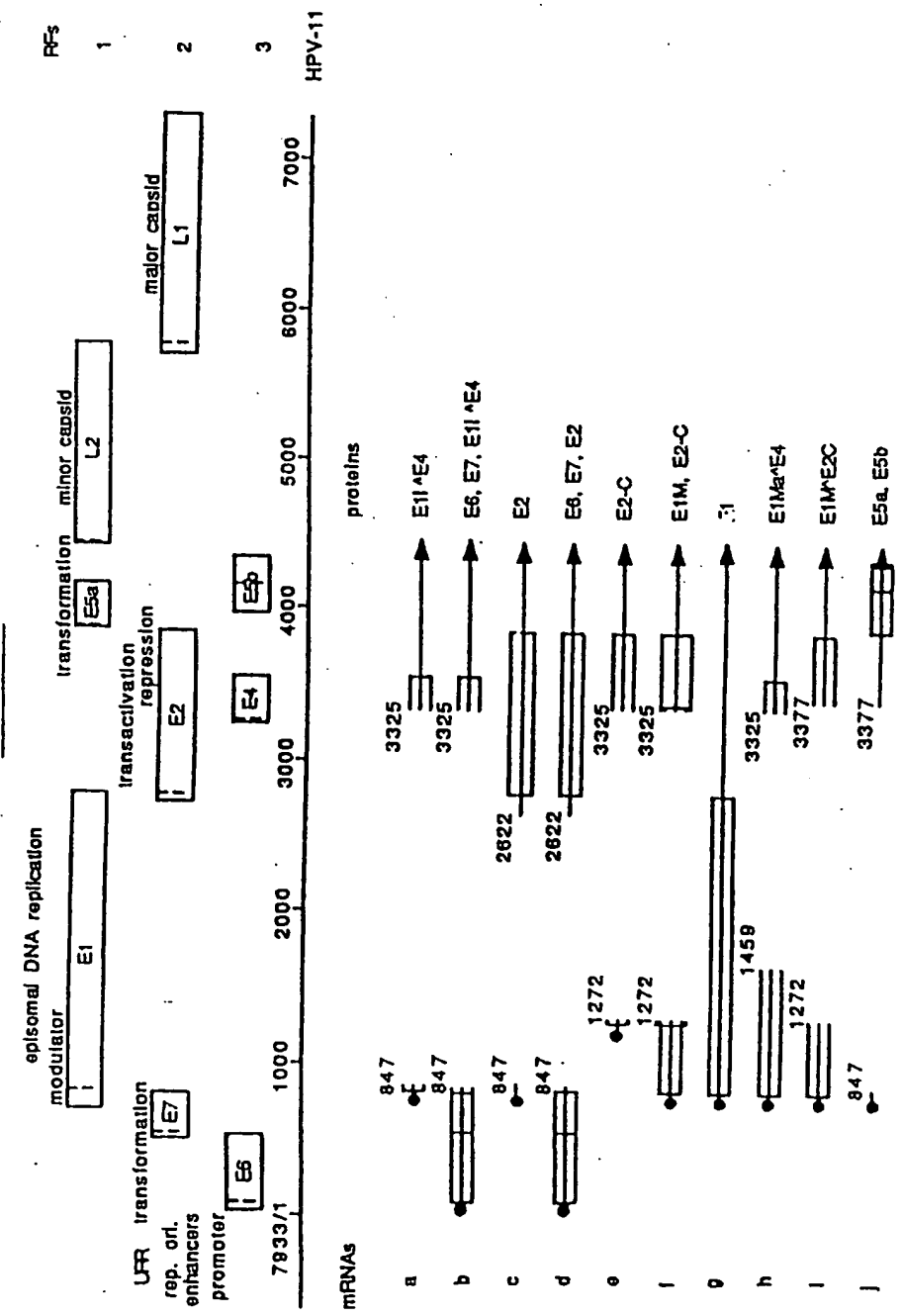


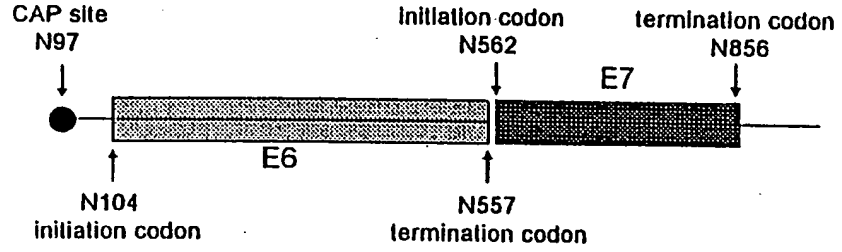
FIGURE 8


RFs
1
2
3
HPV-11
episomal DNA replication
modulator
E1
transformation
E5a
minor capsid
L2
major capsid
L1
transactivation
repression
E2
E4
E5b
URR transformation
rep. ori.
enhancers
promoter
E7
E6
7933/1
1000
2000
3000
4000
5000
6000
7000
mRNAs
proteins
847
3325
E1I^E4
E6, E7, E1I^E4
2622
E2
E8, E7, E2
1272
E2-C
E1M, E2-C
1459
E1Ma^E4
E1M^E2C
E5a, E5b
a
b
c
d
e
f
g
h
i
j

# *Structure of E6/E7 transcripts found in HPV-16 transformed CaSki cells*

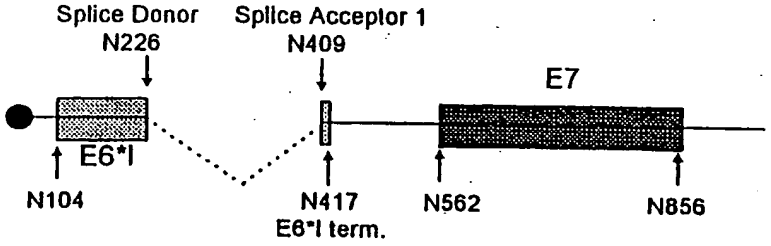
E6/E7 mRNA

Fig. 9A




E6*I/E7 mRNA

Fig. 9B




E6*II/E7 mRNA

Fig. 9C

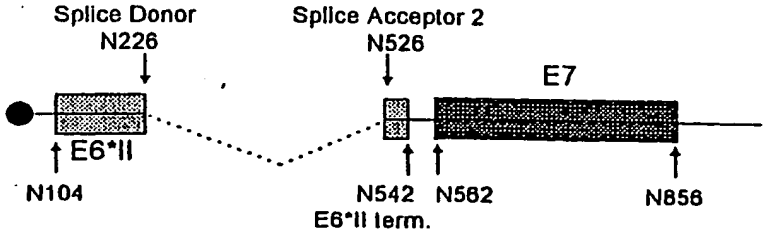
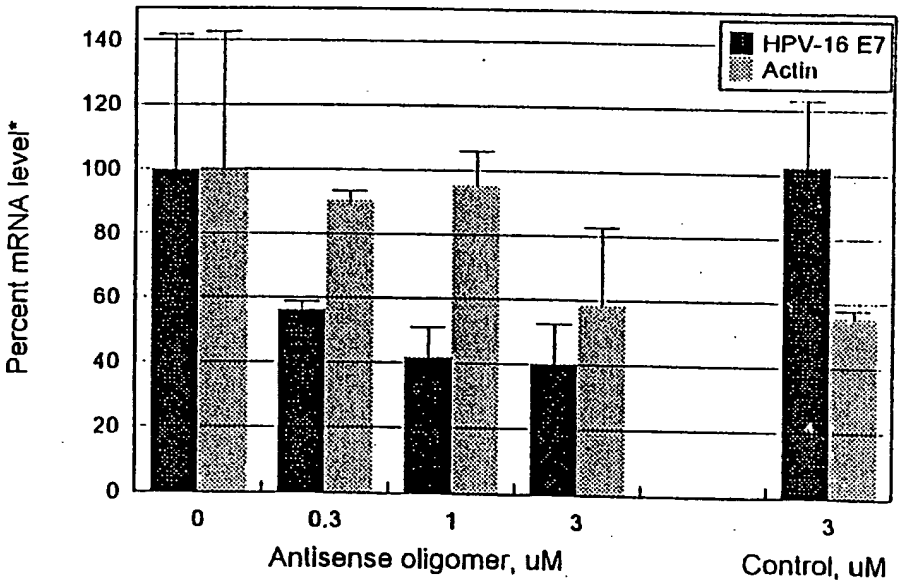


Figure 10

# *Downregulation of HPV-16 E6/E7 mRNA levels in HPV-transformed CaSki cells*

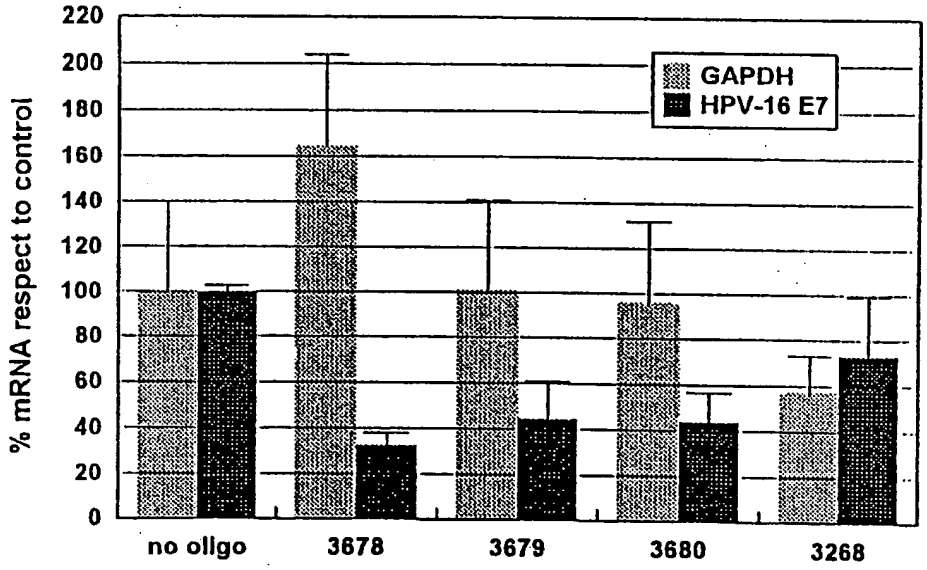
Dose-response with oligomer 3678, [RpMP/DE][PS]5[RpMP/DE]




* Compared to cells treated only with Lipofectamine

Figure 11

*Downregulation of HPV-16 E6/E7 mRNA levels in HPV-transformed CaSki cells treated with [Rp-MP/DE][PS][Rp-MP/DE] oligomers*




**1.0 uM**

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05179

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 31/70; C07H 21/00; C12N 15/11
'S CL :435/6, 91.1; 536/23.1, 24.5; 514/44

A. ·ding to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1; 536 23.1, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG: BIOSIS, MEDLINE, CAS, Derwent Biotechnology Abstracts

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO, A, 93/20095 (CROOKE ET AL.) 14 October 1993, see entire document. | 1-12, 16-26, 28-36, 39-42, 45-53 |
| Y | WO, A, 91/08313 (CROOKE ET AL.) 13 June 1991, see entire document. | 1-12, 16-26, 28-36, 39-42, 45-53 |
| Y | Int. J. Cancer, Vol. 51, No. 5, issued 09 July 1992, Von Knebel Boeberitz et al., "Inhibition of tumorigenicity of cervical cancer cells in nude mice by HPV E6-E7 anti-sense RNA", pages 831-834, see entire document. | 1-12, 16-26, 28-36, 39-42, 45-53 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 11 JULY 1995 | 09 AUG 1995 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>John L. LeGUYADER<br>Telephone No. (703) 308-0196 |

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05179

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Clinical Research, Vol. 39, No. 4, issued 1991, Cowsert et al., "Antisense oligonucleotides as inhibitors of papillomavirus", page 818A, see entire abstract. | 1-12, 16-26, 28-36, 39-42, 45-53 |
| Y | Nucleic Acids Research, Vol. 19, No. 15, issued 1991, Storey et al., "Anti-sense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16", pages 4109-4114, see entire document. | 1-12, 16-26, 28-36, 39-42, 45-53 |
| Y | Antimicrobial Agents and Chemotherapy, Vol. 37, No. 2, issued Feb. 1993, Cowsert et al., "In vitro evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: potential treatment for genital warts", pages 171-177, see entire document. | 1-12, 16-26, 28-36, 39-42, 45-53 |